THE RESPIRATORY NADH DEHYDROGENASE OF *ESCHERICHIA COLI*

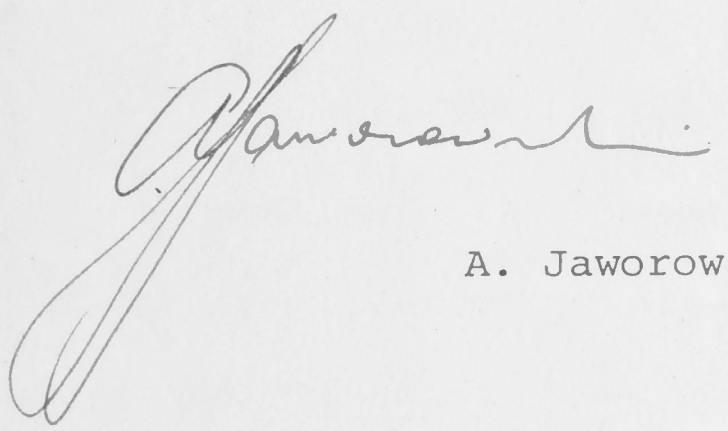
by

Anthony Jaworowski

A thesis submitted for the degree of
Doctor of Philosophy at the
Australian National University, 1980

STATEMENT

All the experimental work reported in this thesis was performed by the author, unless specifically stated otherwise in the text. The work reported in Chapter 5 was done in collaboration with Dr D.C. Shaw.



A. Jaworowski

4/9/80

PREFACE

This thesis describes the results of research carried out in the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, from February, 1977, to September, 1980. These studies were carried out while in receipt of a Commonwealth Postgraduate Award.

Abbreviations used in this thesis without definition:

S.E.	standard error
SDS	sodium dodecyl sulphate
EDTA	ethylenediaminetetraacetic acid
TPCK	L-1-(*p*-toluenesulphonyl)amido-2-phenylethylchloromethyl ketone
CNBr	cyanogen bromide
PTH	phenylthiohydantoin
Ap^r	ampicillin resistance
Tc^r	tetracycline resistance
$tRNA^{f\text{-}met}$	initiation tRNA

Various systems of nomenclature have been used in the literature for the components of the respiratory chain discussed in this thesis. The following nomenclature has been adopted which, although not totally satisfactory, has been imposed in part by past usage.

NADH dehydrogenase (1.6.99.1); any enzyme (complex) catalyzing the oxidation of NADH by artificial electron acceptors. Primary dehydrogenase; the first enzyme in the putative respiratory complex containing the catalytic site for NADH oxidation. Respiratory NADH dehydrogenase

complex; the putative complex catalyzing the transfer of electrons between NADH and ubiquinone *in vivo*. Where specific enzyme catalyzed reactions or the enzymes catalyzing these reactions are meant, they are named according to I.U.B. rules, e.g. NADH:ubiquinone oxido-reductase (1.6.5.3).

ABSTRACT

Membrane particles prepared from wild-type strains of *E. coli* were solubilized with 3% (w/v) potassium cholate and 1M KCl. Chromatography of the solubilized material on hydroxylapatite revealed a single major peak of NADH:ubiquinone oxidoreductase activity. This peak of activity was identified as the respiratory NADH dehydrogenase since it is absent from chromatograms of cholate-solubilized membrane particles prepared from the *ndh* mutant strain IY12, which possesses an inactive NADH dehydrogenase complex (Young, I.G. & Wallace, B.J. (1976) Biochim. Biophys. Acta *449*, 376-385). Several other NADH dehydrogenases, assayed with potassium ferricyanide as electron acceptor, were also resolved by hydroxylapatite column chromatography, but their presence in IY12 column profiles suggests that they are not derived from the respiratory enzyme.

The hybrid plasmid pIY1 (Young, I.G., Jaworowski, A. & Poulis, M.I. (1978) Gene *4*, 25-36) was shown to carry the gene coding for the respiratory NADH dehydrogenase. Strains carrying this plasmid were found to possess amplified levels of NADH:ubiquinone oxidoreductase and NADH oxidase activity in their cell membranes. Methods were developed to further amplify the levels of membrane-bound NADH:ubiquinone oxidoreductase, in strains carrying pIY1, over fifty-fold relative to wild-type levels. These methods should prove generally applicable to the amplification of membrane enzyme levels *in vivo*, being of value both in facilitating the purification of minor membrane components, and in metabolic

studies where being able to modulate the levels of enzymes *in vivo* should give valuable information on the metabolic pathways they are involved in.

The respiratory NADH dehydrogenase was purified to homogeneity from amplified membrane particles as a lipoprotein complex. This preparation possesses the highest reported NADH:ubiquinone oxidoreductase activity; 530 units mg^{-1}. It is postulated that this enzyme contains all the components associated with electron transfer between NADH and ubiquinone *in vivo* and, if so, this is the first highly purified preparation of the NADH: ubiquinone oxidoreductase segment of the respiratory chain, from any source. The enzyme consists of a single subunit of MW ~45,000 with one molecule of FAD per polypeptide chain. No Fe or acid-labile S was detected. The enzyme is in a reconstitutively active form, and a cyanide-sensitive NADH oxidase can be reconstituted from the pure enzyme and *ndh* mutant membrane particles.

In preliminary protein chemical studies, the sequence of the first 20 amino acid residues was determined; data which was subsequently used to locate the structural gene in the complete nucleic acid sequence of the cloned DNA fragment containing the *ndh* gene. Evidence was obtained for the existence of unprocessed enzyme in the preparation having an amino-terminal formyl-methionine; this has important implications with respect to initiation of translation of this particular gene product.

The hybrid plasmid pIY2 (Young, I.G., Jaworowski, A. & Poulis, M.I. (1978) Gene *4*, 25-36) was shown to probably carry the gene coding for the respiratory D-lactate

dehydrogenase flavoprotein, and strains carrying pIY2 possess amplified levels of D-lactate oxidase. A novel pathway for NADH oxidation in strains carrying pIY2 is proposed to explain how this plasmid is able to complement *ndh* mutants.

This work illustrates new genetic and molecular biological approaches which will help to solve difficult problems in the enzymology of membrane-bound systems. A system has been characterized and developed which, by its simplicity and flexibility, should be an excellent model system for probing questions concerning membrane-bound enzymes and membrane proteins in general.

TABLE OF CONTENTS

		Page
Statement		i
Acknowledgements		ii
Preface		iii
Abstract		v
CHAPTER 1.	Introduction.	1
	Organization of Thesis	1
	The Aerobic Respiratory Chain of *E. coli*	2
	The Involvement of Quinones in Electron Transport	7
	Mitochondrial Respiratory Dehydrogenases	9
	Respiratory Dehydrogenases from *E. coli*	10
	The Respiratory NADH dehydrogenase of *E. coli*	19
	Energy Conservation Associated with the NADH:ubiquinone Oxidoreductase Segment of the Respiratory Chain	23
CHAPTER 2.	Identification and Partial Purification of the Respiratory NADH dehydrogenase from the Wild-Type Strain IY13.	26
	Introduction	26
	Experimental	29
	Chemicals	29
	Bacterial Strains and Plasmids	29
	Growth Media	30
	Growth of Bacteria and Preparation of Membranes	30
	Solubilization of Membranes and Hydroxylapatite Column Chromatography	34

TABLE OF CONTENTS Continued

		Page
	Enzyme Assays	35
	Preparation of Hydroxylapatite	36
	SDS-polyacrylamide Gel Electrophoresis	37
	Protein Estimation	37
	Results and Discussion	37
	Respiratory Activities in *ndh* Mutant and Control Strains	37
	Cyanide Sensitivity of the Residual NADH oxidase in *ndh* Mutant Membrane Particles	38
	Solubilization and Chromatography of NADH:ubiquinone Oxidoreductase from Wild-Type Membrane Particles	40
	Attempted Purification of NADH: ubiquinone Oxidoreductase from Strain IY13	50
	Summary	52
CHAPTER 3.	Amplification of the Respiratory NADH dehydrogenase in Membrane Particles	54
	Introduction	54
	Experimental	57
	Media and Growth of Bacteria	57
	Growth Tests	58
	Amino Acid Starvation Experiments with IY35	59
	Recovery of Growth after Chloramphenicol Inhibition	59
	Determination of Optimum Inhibitory Concentration of Chloramphenicol	59
	Amplification of IY35 with Chloramphenicol	60

TABLE OF CONTENTS Continued

	Page
Preparation of Membrane Particles and Assay Procedures	60
Quinone Estimations on Whole Cells	61
Determination of Cytochrome Levels in Membrane Particles	62
Low Temperature Cytochrome Spectra	63
Results and Discussion	63
Respiratory Activities in Membrane Particles Prepared from Strain IY35	63
Amplification of Plasmid Copy-Number	66
Amplification of Enzyme Levels by the Use of Amino Acid Starvation	66
Amplification of Enzyme Levels with Chloramphenicol	67
Complementation of *Ndh* in IY36	78
Growth Characteristics of IY12, IY13, IY35 and IY36	82
Quinone and Cytochrome Content of *Ndh* Mutant, Wild-Type and Plasmid Strains	85
A General Method for the Amplification of Membrane-Bound Proteins *in vivo*	90
Summary	92
CHAPTER 4. Purification and Properties of the Respiratory NADH dehydrogenase from Genetically-Amplified Strains	94
Introduction	94
Experimental	95
Media and Growth of Bacteria	95

TABLE OF CONTENTS Continued

	Page
Purification of NADH: ubiquinone Oxidoreductase	96
Large-Scale Growth of Bacteria	96
Large-Scale Enzyme Purification	96
SDS-polyacrylamide Gel Electrophresis	97
Determination of FAD	98
Extraction and Estimation of Lipid	99
Reconstitution of *Ndh* Mutant Membrane Particles	100
Metal Analyses and Other Assays	101
Results and Discussion	102
Purification of NADH:ubiquinone Oxidoreductase from Chloramphenicol Amplified IY35 Cultures	102
Estimation of the Subunit Molecular Weight of NADH: ubiquinone Oxidoreductase	111
Lipid Content of the NADH: ubiquinone Oxidoreductase Preparation	113
Identification and Quantification of the Flavin Prosthetic Group of NADH:ubiquinone Oxido-reductase	116
Visible and Ultraviolet Spectra of NADH:ubiquinone Oxido-reductase	121
Reconstitution of NADH Oxidase in Membrane Particles Prepared from *Ndh* Mutants	124
Large-Scale Purification of NADH:ubiquinone Oxidoreductase Using Strain IY85	136

TABLE OF CONTENTS Continued

		Page
	Analysis of Metal Content of NADH:ubiquinone Oxido-reductase Preparations	138
	Summary	140
CHAPTER 5.	Protein Chemical Studies on the Respiratory NADH dehydrogenase	143
	Introduction	143
	Experimental	144
	Amino Acid Composition of NADH: ubiquinone Oxidoreductase	144
	Dual Column Amino Acid Analysis	145
	Peptide Mapping of NADH: ubiquinone Oxidoreductase	
	CNBr Cleavage	146
	Detection of Formyl-homoserine	148
	Determination of Amino Terminal Sequence	148
	Results and Discussion	149
	Amino Acid Composition of NADH: ubiquinone Oxidoreductase	149
	Peptide Mapping of NADH: ubiquinone Oxidoreductase	155
	Amino Acid Analysis of CNBr and Tryptic Peptides	161
	N-terminal Amino Acid Sequence of NADH:ubiquinone Oxidoreductase	167
	Demonstration of the Amino-Terminal Formyl-Methionine	173
	Summary	176
CHAPTER 6.	General Discussion	178
REFERENCES		195

CHAPTER 1.

Introduction

Organisation of Thesis.

In this thesis the purification and properties of the respiratory NADH dehydrogenase complex from *E. coli* is presented. Chapter 2 details the attempts to purify the complex as a ubiquinone reductase following solubilization of wild-type membrane particles. Although this was not achieved, it was possible to identify the NADH:ubiquinone oxidoreductase which was solubilized, as the respiratory NADH dehydrogenase by the use of an *ndh* mutant strain. To facilitate the purification of the NADH:ubiquinone oxidoreductase, techniques were developed to amplify the levels of the respiratory dehydrogenase in the membrane by using gene-cloning techniques. These experiments are described in Chapter 3. With the use of the genetically-amplified membrane particles, developed in Chapter 3, and the purification procedures described in Chapter 2, it was possible to purify the respiratory NADH dehydrogenase to homogeneity and characterize the preparation. These results are presented in Chapter 4. Some protein chemical studies on the pure enzyme are presented in Chapter 5; though not complete, they point to the existence of unique properties in the initiation of translation of this particular gene product.

In this introduction, the details of the respiratory pathway, that this enzyme is an important part of, are presented. The properties of the mitochondrial NADH and succinate dehydrogenase complexes are discussed since these two systems are the only well-studied respiratory-chain-linked ubiquinone reductases. There has been little,

if any, enzymology on the quinone reductase components of the *E. coli* respiratory chain: however a survey on the work done in purifying soluble dehydrogenase preparations active with artificial electron acceptors, and derived from respiratory components, is presented, concentrating on those attempts to purify the respiratory NADH dehydrogenase. An associated reaction of the NADH dehydrogenase complex in *E. coli* is thought to be that of energy conservation coupled to quinone reduction, although this has not been universally accepted at present. A summary of the experimental evidence for such a reaction is presented.

The Aerobic Respiratory Chain of *E. coli*.

Escherichia coli is a facultative anaerobe. It derives most of its energy from respiration by using O_2 as terminal electron acceptor, under conditions of aerobic growth, and fumarate or NO_3^- under conditions of anaerobic growth (Haddock & Jones, 1977). The respiratory chain can be seen as a more complicated system compared to that of the mitochondrion due to the variety of terminal electron acceptors and the larger number of various dehydrogenases (inducible and non-inducible) which feed-in to the respiratory chain. Only the aerobic respiratory chain will be considered here.

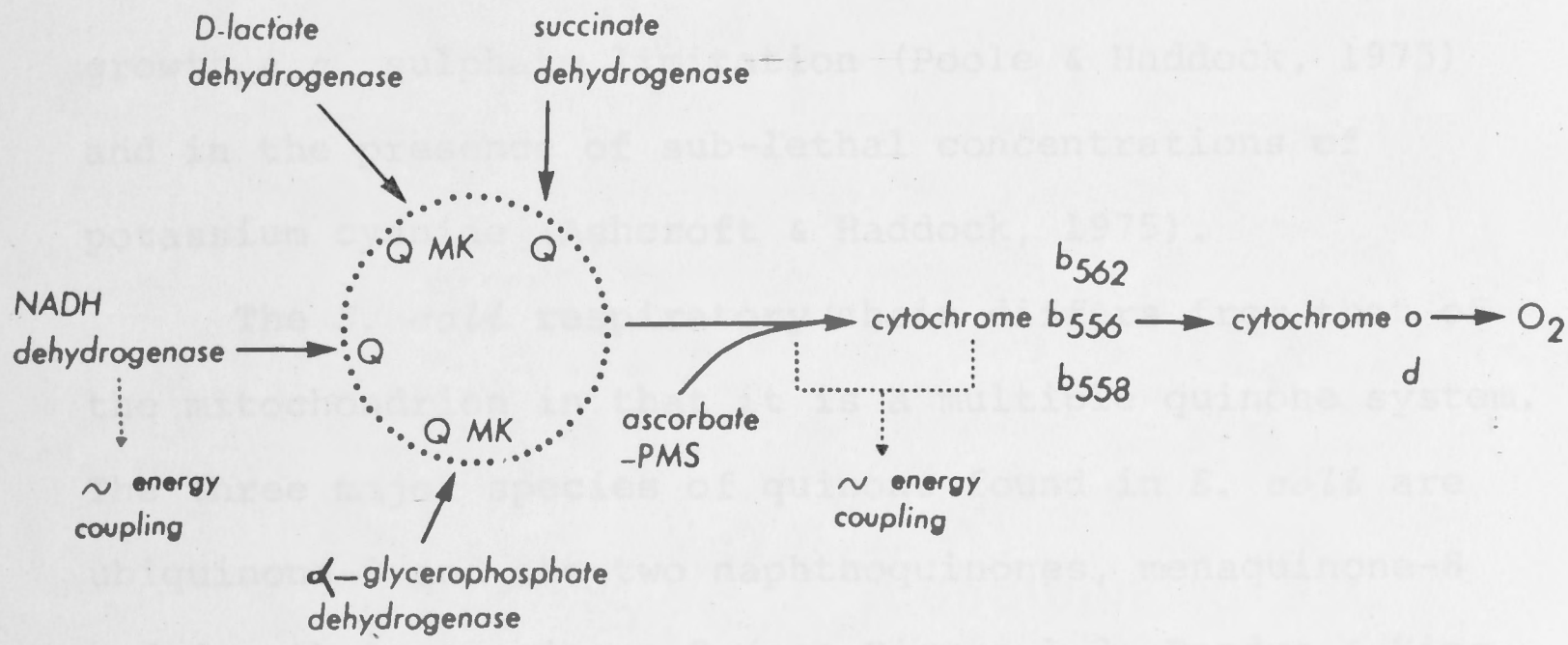
Despite obvious differences in detail, the aerobic respiratory chain of *E. coli* is basically similar to that of the mitochondrion in that in both cases there are a series of substrate-specific dehydrogenases which catalyze the transfer of electrons to a quinone pool, and

ultimately to molecular O_2 via a cytochrome chain (see Figure 1-1). The major non-inducible respiratory-linked dehydrogenases are the NADH, succinate and D-lactate dehydrogenases. In addition, growth on certain carbon sources induces the synthesis of specific respiratory-linked dehydrogenases capable of metabolizing that substrate, *e.g.* L-lactate (Kline & Mahler, 1965; Futai & Kimura, 1977), D-alanine (Raunio & Jenkins, 1973; Olsiewski *et al.*, 1980), proline (Frank & Rybicki, 1961; Scarpulla & Soffer, 1978) and glycerol (Lin *et al.*, 1962; Kistler *et al.*, 1969; Schryvers *et al.*, 1978).

There are five major cytochromes synthesized aerobically which are membrane-bound and thought to be involved in respiration. These are cytochromes b_{556}, b_{558}, b_{562}, *o* and *d* (Shipp, 1972). Cytochrome b_{562} is only partially membrane-bound, and a considerable proportion of this cytochrome is found in the cytoplasm (Ashcroft & Haddock, 1975; Hagaki & Hager, 1966). Its involvement in respiration is uncertain. Cytochromes *o* and b_{556} are the major cytochromes found under conditions of vigorous aeration. Cytochrome *o*, which is a *b*-type cytochrome, acts as a terminal oxidase (Haddock *et al.*, 1976). It exhibits a high affinity for KCN (Pudek & Bragg, 1974) and has been shown to be kinetically competent to act as a terminal oxidase under conditions of high O_2 tension (Haddock *et al.*, 1976).

There have been reports that under conditions of low O_2 tension, when cultures approach late exponential and stationary phases, the content of cytochromes b_{558} and *d* increase in the membrane (Pudek & Bragg, 1975; Shipp,

FIGURE 1-1. The Aerobic Respiratory Chain of *E. coli*.



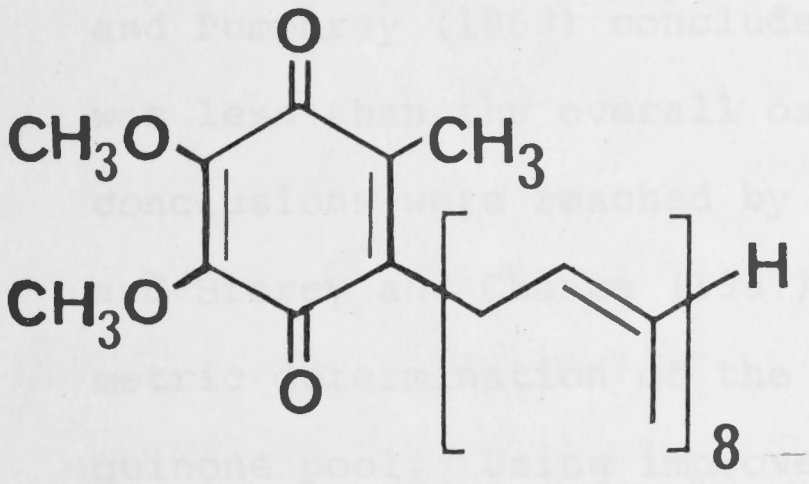
A schematic representation of the aerobic respiratory chain of *E. coli* showing the postulated sites of energy conservation: after Wallace & Young (1977b).

1972). It has been postulated that under these conditions an alternative cytochrome chain is synthesized consisting of these two cytochromes, with cytochrome *d* acting as a terminal oxidase. How this may function as a response to lowered O_2 tension is suggested by the higher affinity of cytochrome *d*, compared to cytochrome *o*, for O_2, as indicated by its lower sensitivity to KCN (Haddock & Jones, 1977; Pudek & Bragg, 1974; Pudek & Bragg, 1975).

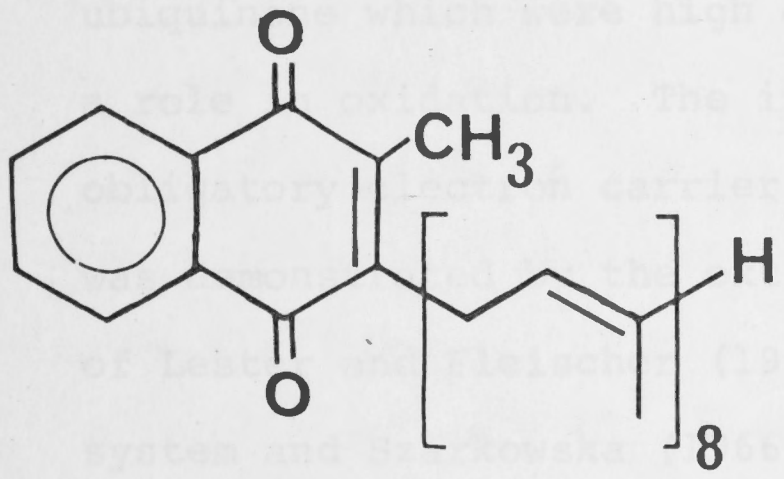
Increased synthesis of cytochromes b_{558} and *d* have also been observed under conditions of sub-optimal aerobic growth, *e.g.* sulphate limitation (Poole & Haddock, 1975) and in the presence of sub-lethal concentrations of potassium cyanide (Ashcroft & Haddock, 1975).

The *E. coli* respiratory chain differs from that of the mitochondrion in that it is a multiple quinone system. The three major species of quinone found in *E. coli* are ubiquinone-8 and the two naphthoquinones, menaquinone-8 and demethylmenaquinone-8 (see Figure 1-2; Pandya & King, 1966; Wallace & Young, 1977(b)). Ubiquinone-8 is the predominant quinone species present in aerobically grown *E. coli*, whereas the levels of the two napthoquinones are higher in anaerobically-grown cells (Bishop *et al.*, 1962; Wallace & Young, 1977(b); Alexander & Young, 1978(a) & (b)). The levels of menaquinone-8 have been observed to increase when cells are grown in the presence of sub-lethal concentrations of KCN (Ashcroft & Haddock, 1975) and where there exists an impairment in the activity of the respiratory chain, *e.g.* in ubiquinone-deficient (Cox *et al.*, 1970) or haem-deficient (Haddock & Schairer, 1973) strains.

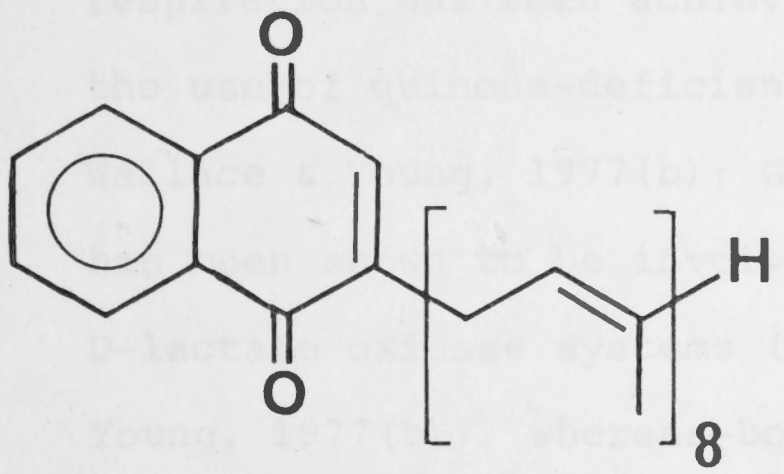
FIGURE 1-2. Structure of the Major Quinone Species in *E. coli*.



Ubiquinone-8



Menaquinone-8



Demethylmenaquinone-8

The Involvement of Quinones in Electron Transport.

There was some early doubt concerning the involvement of ubiquinone as an obligatory electron carrier in mitochondrial respiration. Using chemical extraction methods to determine the extent of reduction of endogenous ubiquinone by succinate and NADH, Redfearn and Pumphrey (1960) concluded that the rate of reduction was less than the overall oxidase rate. Similar conclusions were reached by Chance and Redfearn (1961) and Storey and Chance (1967) using direct spectrophotometric determination of the extent of reduction of the quinone pool. Using improved chemical extraction and spectrophotometric techniques, Kröger and Klingenberg (1966) obtained rates of reduction of the endogenous ubiquinone which were high enough to be consistent with a role in oxidation. The involvement of ubiquinone as an obligatory electron carrier in mitochondrial respiration was demonstrated by the extraction/reactivation studies of Lester and Fleischer (1959) for the succinate oxidase system and Szarkowska (1966) and Ernster *et al.*, (1969(a) & (b)) for the NADH oxidase. In the case of *E. coli*, demonstration of the involvement of ubiquinone in respiration has been achieved in elegant experiments with the use of quinone-deficient strains (Cox *et al.*, 1970; Wallace & Young, 1977(b); Gibson & Cox, 1973). Ubiquinone has been shown to be involved in the NADH, succinate and D-lactate oxidase systems (Cox *et al.*, 1970; Wallace & Young, 1977(b)), whereas both ubiquinone and menaquinone may participate in electron transport from α-glycerophosphate and, to a lesser extent, D-lactate (Wallace &

Young, 1977(b)). These studies have discounted earlier reports that ubiquinone is not involved in NADH oxidation in *E. coli*. Irradiation of membrane particles at 360nm led to destruction of endogenous quinone and loss of NADH and succinate oxidase activity (Kashket & Brodie, 1963(b)). Reactivation experiments with vitamin K_2 and ubiquinone-10 led to the conclusion that ubiquinone is involved in succinate oxidase and menaquinone in NADH oxidase. Bragg & Hou (1967(b)) prepared membrane particles which contained a very low level of endogenous ubiquinone. From these particles they extracted two menadione reductases. One of these enzymes was not active towards ubiquinone and was assumed to be the respiratory NADH dehydrogenase (see below). They concluded that the major pathway of NADH oxidation in *E. coli* is not quinone dependent.

Two models of quinone involvement in *E. coli* respiration have been proposed. Cox *et al.* (1970) and Downie and Cox (1978) have postulated that ubiquinone functions at two sites; between the NADH dehydrogenase and cytochrome b_{562} and at a second site between cytochrome b_{556} and the terminal oxidases (Downie & Cox, 1978). In a model based upon that of Kröger & Klingenberg (Kröger *et al.*, 1973(a) & (b); Kröger & Klingenberg, 1970), Wallace and Young (1977(b)) have proposed that the quinones act as a pool of mobile electron carriers between the dehydrogenases and a common cytochrome chain. The specificity of each oxidase system with respect to the various quinones would reside with the substrate-specificity at the active site of each dehydrogenase

complex. Both models include the concept of an NADH dehydrogenase complex capable of reducing ubiquinone *in vivo*.

Mitochondrial Respiratory Dehydrogenases.

There has been a substantial amount of work carried out on the various respiratory-linked dehydrogenases of the mitochondrion: *e.g.* L-glycerol-3-phosphate dehydrogenase, choline dehydrogenase, yeast D and L-lactate dehydrogenases, succinate dehydrogenase and NADH dehydrogenase (Hatefi & Stigall, 1976). By far the majority of this work has been carried out on the latter two enzymes, and these will be considered in detail here.

The pioneering work of Hatefi and his co-workers resulted in the resolving of the mitochondrial respiratory chain into a series of lipoprotein complexes capable of interacting stoichiometrically to form a particulate unit with similar activities and inhibition characteristics to the intact respiratory chain (Hatefi & Rieske, 1967). These complexes represent the NADH:ubiquinone oxido-reductase (complex 1), succinate:ubiquinone oxidoreductase (complex 2), ubiquinol:cytochrome c oxidoreductase (complex 3) and cytochrome c oxidase (complex 4) segments of the respiratory chain. They have not only proven valuable in studying the various partial reactions of the respiratory chain, but have also served as the starting material for further purification of respiratory components.

A. Succinate Dehydrogenase Complex.

Complex 2 catalyzes the transfer of electrons between succinate and ubiquinone (Zeigler & Doeg, 1959; Ziegler & Doeg, 1962; Hatefi *et al.*, 1961) with a specific activity of 45 to 55μmol min^{-1} mg^{-1} (at 38^{o}) using ubiquinone-2 as electron acceptor. It contains 5nmoles of covalently-bound flavin per mg protein, and 7 to 8 g-atoms of iron and 7 to 8moles of acid-labile S per mole of flavin. In addition, complex 2 contains a *b*-type cytochrome in approximately equimolar amount to the flavin. This cytochrome, $b_{557.5}$ (Davis *et al.*, 1973) is found exclusively in complex 2 following fractionation of the respiratory chain into its constituent complexes. It is not detectably reduced by succinate in either complex 2 or respiratory particles, and its significance is unknown (however, see below).

Complex 2 has been used as the starting material for the preparation of soluble succinate dehydrogenase using various chaotropic agents (Davis & Hatefi, 1971). The soluble succinate dehydrogenase (assayed with phenazine methosulphate as electron acceptor) has a molecular weight of 97,000 and consists of two distinct subunits of molecular weights ~70,000 and ~27,000. The preparation contains ~10nmoles of covalently-bound FAD mg^{-1} protein (located on the larger subunit) with a flavin:iron:acid-labile S ratio the same as for complex 2 (Davis & Hatefi, 1971; Hatefi & Stigall, 1976).

The FAD was found to be covalently linked to the N-3 nitrogen of a histidyl residue *via* the methyl group at position 8 on the isoalloxazine ring (Hemmerich *et al.*,

1969; Salach *et al.*, 1972). The sequence of a penta-peptide containing the histidyl-8-α-FAD has been obtained (Singer *et al.*, 1973): ser-his-thr-val-ala. The exact number and the nature of the iron-sulphur prosthetic groups in succinate dehydrogenase and succinate:ubiquinone oxidoreductase is still a matter of controversy (Ohnishi *et al.*, 1976; Coles *et al.*, 1979; Albracht, 1980).

The structure of the soluble succinate dehydrogenase from mitochondria is very similar to that purified from *R. rubrum* (Davis & Hatefi, 1971; Hatefi *et al.*, 1972).

Recently, a preparation of succinate:ubiquinone oxidoreductase was purified following triton X-100 solubilization of *N.crassa* mitochondrial particles (Weiss & Kolb, 1979). This preparation contained three subunits (molecular weights 72,000, 28,000 and ~14,000); two subunits analogous to those of the soluble succinate dehydrogenase from mammalian mitochondria and *R. rubrum*, and one *b*-type cytochrome. A similar preparation has been purified from the cytoplasmic membrane of *B. subtilis* (Hederstedt *et al.*, 1979) by immunoprecipitation of triton X-100 solubilized membranes. It is suggested that succinate dehydrogenase is attached to the membrane-bound cytochrome *b via* the smaller (molecular weight 28,000) iron-protein subunit (ibid.).

The scheme of electron transport between succinate and ubiquinone is complicated by the reports of Yu *et al.* (1977(a) & (b)), who have isolated a protein from mammalian mitochondria (QPs) (molecular weight, 15,000), which reconstitutes a thenoyltrifluoroacetone (TTFA)-sensitive

succinate:ubiquinone oxidoreductase activity when added to soluble succinate dehydrogenase. This protein is assumed to act as a ubiquinone binding protein (ibid.), and possibly even to allow electron transport to occur between the soluble succinate dehydrogenase and ubiquinone outside of the lipid phase (Yu & Yu, 1980). However, the preparation of QPs does not contain iron or haem and is thought to be distinct from cytochrome *b*.

Succinate:ubiquinone oxidoreductase is the best-characterized respiratory-linked enzyme catalyzing the reduction of ubiquinone, having all of the polypeptides and prosthetic groups involved in catalysis close to being identified.

B. The NADH dehydrogenase Complex.

Complex 1 (see above) catalyzes the transfer of electrons between NADH and ubiquinone; the specific activity with ubiquinone-1 as electron acceptor is 25μmoles min^{-1} mg^{-1} (Merola *et al.*, 1963). The complex contains non-covalently-bound FMN, non-haem iron, acid-labile S, ubiquinone and lipid (Table 1-1).

The reduction of ubiquinone, catalyzed by complex 1, is sensitive to rotenone and piericidin (Hatefi *et al.*, 1962; Hatefi & Stigall, 1976) and, by this criterion, is similar to the NADH-dependent reduction of ubiquinone-1 by mitochondrial particles and to the reduction of endogenous ubiquinone during NADH oxidation.

Complex 1 has been reconstituted with artificial phospholipid vesicles and shown to couple protein gradient formation to the reduction of exogenous ubiquinone-1 (Ragan & Hinkle, 1975). The reconstituted vesicles could

TABLE 1-1. Composition of Complex 1

Component	Content
FMN (acid extractable)	1.4-1.5nmoles mg^{-1}
non-haem iron	23-26ng atoms mg^{-1}
acid-labile sulphide	23-26nmoles mg^{-1}
ubiquinone-10	4.2-4.5nmoles mg^{-1}
lipid	0.22mg mg^{-1}

(Hatefi *et al.*, 1962)

also support the uptake of a lipophilic ion, tetraphenylboron. This complex is therefore an intact preparation of the NADH:ubiquinone oxidoreductase segment of the respiratory chain and the energy conservation site associated with it.

SDS-polyacrylamide gel electrophoresis reveals the presence of at least 10 different polypeptides (Hatefi & Stempel, 1969). The polypeptide composition of complex 1 has recently been extensively studied (Ragan, 1976(b); Crowder & Ragan, 1977; Heron *et al.*, 1979) and up to 26 different polypeptides have been resolved. This entity has been assumed to be a definite stoichiometic complex (Heron *et al.*, 1979; Ragan, 1976(b)) but as to how many of these polypeptides are actually involved in electron transport, or even the associated energy conservation reaction, is unclear.

Complex 1 has been shown, by EPR spectroscopy, to possess four distinct iron-sulphur prosthetic groups (Orme-Johnson *et al.*, 1974). All four centres are rapidly reduced by NADH: the order of this reduction was obtained kinetically by measuring the rate of appearance of the various signals using 3-acetylpyridine NADH as substrate. The number of iron-sulphur prosthetic groups is by no means agreed upon, however (Ohnishi *et al.*, 1974; Ohnishi, 1975).

Several soluble NADH dehydrogenases have been purified from either complex 1 or mitochondrial particles. These preparations fall into two groups; the high molecular weight (type I) dehydrogenase (Ringler *et al.*, 1963) and the low molecular weight (type II) dehydrogenases (Mahler

et al., 1952; Pharo *et al.*, 1966; King & Howard, 1962; Mahler, 1961; Kumar *et al.*, 1968; Hatefi & Stempel, 1969). The former preparation has a molecular weight of ~800,000, and was obtained by phospholipase digestion of membrane particles at 30°. Solubilization with phospholipase at 37° or by exposure to 9% ethanol at 43°, pH5 (heat-acid-ethanol) produces the latter which has a molecular weight of ~80,000. Exhaustive work by Singer and co-workers has demonstrated that apparently different preparations of the latter enzyme are all basically identical. Hatefi and Stempel (1969) and Galante and Hatefi (1979) have purified, and extensively characterized, a type II dehydrogenase prepared by chaotropic resolution of complex 1. The latter preparation contains three subunits of molecular weight 51,000, 24,000 and 9-10,000. It contains FMN (13.5nmoles mg^{-1} protein), non-haem iron (74ng atoms mg^{-1} protein) and acid-labile S (72 nmoles mg^{-1} protein). The ratio of non-haem iron to flavin suggests the presence of two (and possibly three) iron-sulphur clusters. The structure is reminiscent of that of succinate dehydrogenase (see above). Amino acid compositions have been obtained for each subunit (Galante & Hatefi, 1979).

A controversy has arisen in the literature concerning the significance of the type II dehydrogenase preparations, especially regards their high ubiquinone reductase activity. These arguments are too lengthy to consider here, in depth, and the reader is directed to various reviews propounding the different points of view (Singer & Gutman, 1971; Hatefi & Stigall, 1976; see also Ragan, 1976). Basically, the high ubiquinone reductase activity of type II dehydrogenase has been considered

artifactual, and unrelated to the physiological ubiquinone reductase activity (Singer & Gutman, 1971). The major experimental evidence supporting this is (1) The rotenone sensitivity of the ubiquinone reductase activity of type II dehydrogenases differs from that of membrane particles or complex 1. (2) Transformation of type I to type II dehydrogenase occurs under the same conditions as the solubilization of type II dehydrogenase from the membrane: since the type I dehydrogenase possesses negligible ubiquinone reductase activity, that of the type II dehydrogenase is generated by solubilization.

The physiological reduction of ubiquinone has been shown to require phospholipid (Ragan & Racker, 1973; Heron *et al.*, 1977). The soluble dehydrogenase cannot therefore be expected to catalyze this reaction. What is more relevant, however, is (1) whether ubiquinone accepts electrons from the same catalytic site (*i.e.* prosthetic group) on the dehydrogenase and in the membrane, and (2) whether any components involved in electron transport between NADH and ubiquinone have been lost or denatured during preparation. These questions have yet to be answered.

Respiratory Dehydrogenases from *E. coli*.

Comparatively little enzymology has been carried out on the enzyme components of the aerobic respiratory chain of *E. coli* compared to those of the mitochondrion.

D-lactate dehydrogenase has been purified from *E. coli* ML (Futai, 1973; Kohn & Kaback, 1973) and *E. coli*

W (Pratt *et al.*, 1979). Futai (1973) solubilized membrane particles with sodium deoxycholate and purified the solubilized enzyme by a series of ammonium sulphate and chloroform precipitations, followed by chromatography on DEAE-sephadex and DEAE-cellulose. The purified enzyme consisted of a single polypeptide of apparent molecular weight 72,000, containing between 0.8 and 1.7eq FAD per subunit. A similar preparation was obtained by Kohn and Kaback (1973), who fractionated crude extracts of *E. coli* with ammonium sulphate, sodium perchlorate and DEAE-cellulose column chromatography, and solubilized the enzyme by DEAE-cellulose column chromatgraphy in the presence of triton X-100. The purified enzyme was reported to consist of a single subunit of apparent molecular weight 73,000 and containing ~leq FAD per subunit. Pratt *et al.* (1979) solubilized membrane particles with sodium deoxycholate then purified D-lactate dehydrogenase by DE-52, Sephadex G-200 and hydroxylapatite column chromatography in the presence of triton X-100 and deoxycholate. The preparation consisted of a single subunit of apparent molecular weight ~75,000. The preparations of Futai (1973) and Kohn and Kaback (1973) were active with D-lactate and, to a lesser extent, L-lactate (12 and 17% respectively under the standard assay conditions used). The enzyme preparations were assayed by measuring phenazine metho-sulphate (PMS) mediated reduction of 3,(4,5-dimethyl-thiazolyl-2-)-2,5-diphenyltetrazolium bromide (MTT); in no case was reactivity towards ubiquinone as electron acceptor reported.

Futai and Kimura (1977) reported the purification of an inducible L-lactate dehydrogenase following solubilization of membrane particles with sodium cholate and chromatography in the presence of Tween 80. The enzyme consisted of a single polypeptide of apparent molecular weight 43,000 and existed as an oligomer of average molecular weight 480,000 in the presence of these detergents. The preparation was shown to contain ~1eq FMN per subunit. The enzymic activity was assayed by measuring the PMS coupled reduction of MTT by L-lactate. D-lactate was inactive as a substrate. No reactivity towards ubiquinone was reported.

Scarpulla and Soffer (1978) reported the solubilization and purification of an inducible proline dehydrogenase which contained a single polypeptide of apparent molecular weight ~124,000. The preparation, which catalyzed the transfer of electrons from L-proline to various artificial dye-acceptors, was shown to have ~0.24eq FAD per subunit, and required reactivation by FAD in the assay for full activity.

Olsiewski *et al.* (1980) have recently reported the partial purification of an inducible D-amino acid dehydrogenase following solubilization of membrane vesicles with triton X-100. SDS-polyacrylamide gel electrophoresis indicates that the purified enzyme consists of two subunits of apparent molecular weights 45,000 and 55,000. The enzyme contains FAD and non-haem iron in the ratio of 1:2 to 3, but the distribution of these prosthetic groups amongst the two subunits has not

been determined. It is not known as yet whether the iron in the preparation is associated with an iron-sulphur prosthetic group. The enzyme preparation possesses a low activity towards ubiquinone-1 as electron acceptor (specific activity = 2.4 units/mg under their standard assay conditions, and with D-alanine as electron donor).

The various attempts to purify the respiratory NADH dehydrogenase from *E. coli* are dealt with elsewhere (see below).

The Respiratory NADH dehydrogenase of *E. coli*.

Various attempts have been made in the past to purify the respiratory NADH dehydrogenase from *E. coli*. Wosilait and Nason (1954) reported the partial purification of a menadione reductase from crude extracts prepared by grinding cells in alumina. This preparation was active with both NADH and, to a lesser extent, NADPH, and was distinguished from the 'quinone reductase' activity (assayed with *p*-benzoquinone as electron acceptor) also found in crude extracts of *E. coli*.

Kashket and Brodie (1963(b)) solubilized an NADH dehydrogenase from small particles, with deoxycholate. The enzyme was not further purified. The preparation could reduce vitamin K_2, menadione and, to a lesser extent, vitamin K_1. The specific activity of the preparation however was very low (0.021μmol min^{-1} mg^{-1} with vitamin K_2 as electron acceptor). From UV-irradiation/reactivation experiments, it was concluded that menaquinone is involved

in NADH oxidation in the small particle fraction (see above). The enzyme solubilized from small particles was differentiated from the quinone reductase activity of either large particles or the cytoplasmic fraction.

Bragg (1965) sonicated *E. coli* suspensions in the presence of deoxycholate, and purified two NADH dehydrogenases which catalyzed the menadione-mediated reduction of O_2 and triphenyltetrazolium chloride (TTC) respectively. Bragg and Hou (1967(a)) solubilized a small particle fraction of *E. coli* with deoxycholate and resolved two menadione reductases (menadione reductases I and II) by calcium phosphate gel chromatography. One of these activities (menadione reductase II) was further purified by DEAE-cellulose column chromatography (Bragg and Hou, 1967(b)) which separated it from a contaminating NADH oxidase activity. Menadione reductases I and II were active with both NADH and NADPH as electron donor. On the basis of sensitivity to various inhibitors, it was concluded that menadione reductase I is the major respiratory dehydrogenase. The small particle fraction, used as the starting material for the isolation of these enzymes, was found to have very low levels of ubiquinone (Bragg & Hou, 1967(a)) and it was concluded that the major pathway of NADH oxidation is quinone independent.

Gutman *et al.* (1968) prepared and lyophilized a small particle fraction from *E. coli*. When the lyophilized particles were resuspended in distilled water and centrifuged, the yellow supernatant possessed NADH dehydrogenase activity with menadione, potassium ferricyanide and dichlorophenolindophenol as electron

acceptors. No further purification procedures were attempted. The NADH dehydrogenase solubilized in this manner was shown to be different to the soluble diaphorase activity in the cytoplasm by a number of criteria: differences in the apparent K_m for NADH and potassium ferricyanide, and sensitivity to dicoumarol. The enzyme fraction detached from the membrane particles by lyophilization was assumed to be the respiratory NADH dehydrogenase. The preparation was shown to contain approximately equal amounts of both FAD and FMN (0.2 and 0.3nmol mg^{-1} protein, respectively) and non-haem iron and acid-labile sulphur (14 to 16ng atoms and 11 to 13nmoles mg^{-1} protein, respectively).

In the early work described above, no evidence as to the purity of the various preparations was ever given, nor were adequate criteria to identify the various preparations obtained with the respiratory enzyme, even when the presence of contaminating dehydrogenase activities was demonstrated.

The first work which seriously attempted to identify a purified enzyme preparation with the respiratory NADH dehydrogenase, and to give adequate consideration to the problem of contaminating activities, was that of Dancey *et al.*, 1976. They chose osmotically lysed spheroplasts as the starting material for their purification, to try to overcome the problem of the very high levels of NADH dehydrogenase activity in the cytoplasm of *E. coli*. They found that only 10 to 20% of the NADH dehydrogenase activity (measured with dichlorophenolindophenol, DCIP, as electron acceptor) is associated with the membrane

fraction. Spheroplasts, prepared in this manner, have been shown to be substantially free of cytoplasmic contamination (Kaback, 1971). The activity present in the membrane particles was judged to be different to that found in the cytoplasm by the following criteria: (1) The membrane-bound activity is completely inhibited by 10mM AMP, whereas the cytoplasmic activity is only 10% inhibited at this concentration. (2) The membrane-bound activity is highly sensitive to triton X-100. (3) The cytoplasm has a much higher ratio of dehydrogenase to oxidase activity compared to the membranes. (4) There is a very high NADPH dependent DCIP reductase activity in unfractionated cell extracts, but not in the membrane. The level of NADPH dehydrogenase activity was used as a measure of cytoplasmic contamination of the membrane preparation: it was found to be less than 1% of that present in unfractionated cell extracts, confirming the results of Kaback, 1971.

An NADH dehydrogenase was solubilized from spheroplasts with triton X-100 and further purified by ethanol precipitation and DEAE-cellulose, hydroxylapatite and DEAE-agarose column chromatography in the presence of triton X-100. A purification of 9 to 16-fold was achieved over the activity present after solubilization (this was lower, however, when either menadione or potassium ferricyanide were used as substrates). Since the recovery of activity following solubilization was in the range of 30 to 50%, the specific activity of the pure enzyme was only 3 to 8-fold higher than that of the vesicles, in the

DCIP reductase assay. The authors attribute the loss of activity following solubilization to multiple points of interaction of DCIP with the respiratory chain.

The most highly purified preparations contained a single polypeptide of molecular weight 38,000 (estimated densitometrically, on SDS-polyacrylamide gels, to represent 75% of the material in the preparation). The enzyme was estimated to be present at a level equivalent to 2% of the total membrane protein or 0.2% of the total cellular protein. The preparation was NADH-specific and contained no flavin: the enzymic activity showed an absolute requirement for FAD in the assay (Dancey & Shapiro, 1976).

This enzyme was identified as the respiratory NADH dehydrogenase on the basis that it is (1) NADH specific, (2) it shows similar sensitivity to AMP as the membrane-bound NADH oxidase and (3) since antibodies elicited against the pure enzyme inhibit the membrane-bound NADH oxidase.

Energy Conservation Associated with the NADH:ubiquinone Oxidoreductase Segment of the Respiratory Chain.

The existence of a site of energy conservation associated with the NADH:ubiquinone oxidoreductase segment of the mitochondrial respiratory chain has long been assumed from the difference in P/O ratios obtained with NADH-linked substrates and succinate (*e.g.* see Lehninger, 1964). This site of energy conservation, 'site-1', has been demonstrated directly by measuring

uncoupler-sensitive phosphate esterification coupled to NADH-dependent ubiquinone-1 reduction in mitochondrial particles (Schatz & Racker, 1966).

The existence of site 1 in *E. coli* is still a matter of some controversy. Due to the very low P/O ratios obtained with membrane vesicle preparations (*e.g.* see Butlin *et al.*, 1971) no definite statement as to the number of energy coupling sites associated with the various electron transport chains is possible as is the case with mitochondria.

The existence of multiple sites of energy conservation in the *E. coli* respiratory chain has been suggested by measurements of molar growth yields in continuous culture (Harrison & Loveless, 1971) and by whole-cell measurement of phosphate esterification, accompanying O_2 addition to anaerobic suspensions of bacteria (Hempfling, 1970). Both of these approaches have been criticized however (Stouthamer & Bettenhaussen, 1973; van der Beek & Stouthamer, 1973).

Sweetman and Griffiths (1971) reported the preparation of a small particle fraction of *E. coli* which catalyzed ATP-dependent reduction of NAD^+ by succinate. This reaction was sensitive to uncouplers such as pentabromophenol and dicoumarol but not 2,4-dinitrophenol or oligomycin. A similar reaction was demonstrated by Poole and Haddock (1974) in a small particle fraction prepared from glycerol-grown cells but not glucose-grown cells. The activity was sensitive to washing of the particles in low ionic strength buffer and could be demonstrated in particles prepared from ubiquinone-deficient mutant

strains of *E. coli* only after addition of ubiquinone-1. Its presence in haem-deficient strains suggested that this reaction does not involve the cytochrome chain (ibid.).

Lawford and Haddock (1973) demonstrated the presence of two proton translocating segments associated with the NADH oxidase from measurements of uncoupler-sensitive proton translocation in intact, glycerol-grown cells. A $\longrightarrow H^+/O$ ratio of ~4 was obtained with L-malate as a substrate, and ~2 with succinate, D-lactate or glycerol as substrates: assuming a $\longrightarrow H^+$/site ratio of 2, it was concluded that one proton translocation 'loop' is associated with the NADH:ubiquinone oxido-reductase segment of the respiratory chain. From similar measurements on sulphate-limited, glycerol-grown cells (Poole & Haddock, 1975) it was shown that this activity is lost under conditions of sulphate-limitation. It was postulated that under these conditions a non-proton-translocating NADH dehydrogenase is synthesized which may be associated specifically with cytochromes b_{558} and *d* (ibid., Haddock & Jones, 1977).

CHAPTER 2.

Identification and Partial Purification of the Respiratory NADH dehydrogenase from the Wild-Type Strain, IY13

INTRODUCTION

In previous work in this laboratory, a mutant strain of *E. coli*, which is defective in electron transport, was isolated from amongst strains growing poorly on succinate (Young & Wallace, 1976). This strain, AN589, has less than 2% of the wild-type level of NADH oxidase in the plasma membrane, but is unaffected in the levels of D-lactate and succinate oxidases. The mutation has been designated *ndh* and a phenotype established based on the inability of AN589 to grow with mannitol as sole carbon source. Reversion analysis and transductional mapping experiments (I.G. Young, unpublished results, and Young & Wallace, 1976) suggest that the lesion is due to a single point mutation located at minute 22 on the *E. coli* chromosome (Bachmann & Low, 1980). With the above phenotype established, a dozen such mutants were isolated.

The inability of *ndh* mutant strains to grow with succinate as sole carbon source and the fact that they grow poorly on glucose, unless the medium is supplemented with succinate, acetate or casamino acids (a casein hydrolyzate), indicates a defective operation of the tricarboxylic acid cycle. Other evidence indicates that *ndh* mutants have problems in reoxidizing NADH generated mainly during the tricarboxylic acid cycle and glycolysis:

(1) Their inability to grow on mannitol is assumed to be due to the fact that the catabolism of mannitol, compared to that of glucose, generates one extra molecule of NADH in the conversion of mannitol-1-phosphate to fructose-6-phosphate, and, in the absence of the respiratory

NADH oxidase, the cell is unable to regenerate NAD^+ by the subsequent metabolism of fructose-6-phosphate to lactate (Young & Wallace, 1976).

(2) *Ndh* mutant strains, grown on mannitol plus casamino acids, accumulate high concentrations of D-lactate in their culture supernatant (Young, Jaworowski & Poulis, unpublished work). This suggests that in the absence of the respiratory NADH oxidase the reoxidation of NADH coupled to the reduction of pyruvate, and catalyzed by the pyridine nucleotide-linked D-lactate dehydrogenase in the cytoplasm, becomes an important reaction.

Since the *ndh* mutants lack the membrane-bound NADH oxidase activity but not D-lactate or succinate oxidases, it was assumed that the mutation is in the gene coding for a component of the respiratory chain located on the low potential side of the common respiratory components, *i.e.* towards the substrate end of the quinone pool (Young & Wallace, 1976). This was confirmed by experiments showing that the endogenous ubiquinone-8 in membrane particles prepared from AN589 was not reduced in the steady-state after the addition of NADH. The respiratory complex which transfers electrons from NADH to ubiquinone-8 was defined as the NADH dehydrogenase complex (ibid.) and is functionally analogous to the complex I isolated from mitochondria (Hatefi *et al.*, 1962).

NADH dehydrogenase rates in the plasma membrane of AN589, measured with dichlorophenolindophenol as electron acceptor, are 28% of the wild-type level. It has been assumed that because the NADH dehydrogenase activity is not completely abolished, as is the NADH oxidase activity, the

mutation is not in the gene coding for the primary dehydrogenase but in another component of the putative complex - possibly that of the polypeptide which interacts directly with ubiquinone (Young & Wallace, 1976).

Studies using quinone-deficient mutants have established that the natural electron acceptor for the NADH dehydrogenase complex is ubiquinone-8 (Cox *et al.*, 1970; Wallace & Young, 1977). The enzyme is an intrinsic membrane complex in either the inner mitochondrial membrane of eukaryotic cells or the plasma membrane of prokaryotic cells such as *E. coli*. In order to study the structure of the complex which catalyzes the reduction of ubiquinone *in vivo* and, with the eventual aim of studying the mechanism of this and associated reactions, it was decided to try to solubilize the complex, intact, as an NADH:ubiquinone oxidoreductase.

It has not proved possible to study the reaction of this complex with ubiquinone in highly purified preparations from any source. The reactivity towards ubiquinone has been found to be very sensitive to the loss of lipid, and is easily destroyed during solubilization. Large macromolecular aggregates catalyzing the reduction of ubiquinones have however been solubilized from mitochondrial particles using bile salts as detergent (Hatefi *et al.*, 1962). The success in solubilizing the enzyme with its ubiquinone reductase activity intact is related to the fact that the solubilized preparations still retain 20% phospholipid by weight.

In preliminary studies (M.I. Poulis & I.G. Young,

unpublished results) it was found that the enzyme complex from *E. coli* could be solubilized by the use of potassium cholate. In the present chapter the NADH:ubiquinone oxidoreductase solubilized from *E. coli* membrane particles is verified to be the respiratory NADH dehydrogenase complex by comparative studies with *ndh* mutant strains.

EXPERIMENTAL

Chemicals

Cholic acid was obtained from Fluka (Switzerland). Stock solutions (20% (w/v)) are brownish/orange in colour, presumably due to the presence of bile pigments as impurities. For some experiments, cholic acid was twice recrystallized from 50% (v/v) ethanol by a procedure similar to that of Hatefi (1978). Stock solutions of potassium cholate (nominally 20% (w/v)) were prepared by suspending 20g of cholic acid in approx. 80mL of water, and slowly titrating the mixture to pH7.5 with concentrated KOH. The solution was then adjusted to 100mL, filtered, and stored at room temperature. Solutions of recrystallized potassium cholate are completely colourless.

Ubiquinone-1 was the generous gift of Drs. O. Isler (F. Hoffman - La Roche and Co., Basel, Switzerland) and D. Magrath. NADH and NADPH were obtained from P-L Biochemicals (Milwaukee, Wis.) and FAD from Sigma Chemical Co. (St. Louis, Mo.). N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES), sodium salt, was obtained from Calbiochem (La Jolla, Cal.).

Bacterial Strains and Plasmids

All strains used are derivatives of *Escherichia coli*

K-12. The relevant details are presented in Tables 2-1 and 2-2.

Stock cultures were maintained at -20° as frozen suspensions in glycerol-Luria broth medium (20% (w/v) glycerol in Luria broth). Freeze dried cultures of each strain were kept at 4°C as reserve stock.

Growth Media

The mineral-salts medium used has been described elsewhere (Stroobant *et al.*, 1972), and was used at normal strength. The following supplements were sterilized separately and added at the indicated final concentrations to give the minimal medium: mannitol (30mM), L-histidine-HCl (0.15mM), L-isoleucine (0.3mM), L-valine (0.3mM), L-tryptophan (0.2mM) and thiamine-HCl (1μM). Concentrations of other additives used were glucose (as carbon source), 30mM; succinate, 30mM (as carbon source) or 4mM (as supplement); casamino acids, 0.1% (w/v). The complete medium used was brain-heart infusion.

Solid medium contained 2% (w/v) agar (Difco) in minimal medium, plus the relevant additions at the concentrations indicated above.

Growth of Bacteria and Preparation of Membranes

All cultures were grown at 37° in mannitol-minimal medium supplemented with casamino acids unless otherwise specified. Culture turbidity was measured using a Klett-Summerson colorimeter fitted with a blue filter, and is measured in Klett units.

10L batch cultures were grown in 14L New Brunswick glass fermenters with aeration (12L min^{-1}) and stirring

TABLE 2-1 Bacterial Strains

Strain	Relevant Loci	Plasmid	Source or Reference
AN589	*met*B, *ndh*401	-	Young & Wallace, 1976
AN595	*his*, *ilv*, *trp*, *rps*L	-	This laboratory
AN596	" " " " , *ndh*402	-	Derived from AN595 by mutagenesis with NTG[a] (Young & Wallace, 1976)
AN597	" " " " , *ndh*403	-	" " "
AN598	" " " " , *ndh*404	-	" " "
AN599	" " " " , *ndh*405	-	" " "
AN600	" " " " , *ndh*406	-	" " "
AN601	" " " " , *ndh*407	-	" " "
AN602	" " " " , *ndh*408	-	" " "
AN603	" " " " , *ndh*409	-	" " "
AN604	" " " " , *ndh*410	-	" " "
AN605	" " " " , *ndh*411	-	" " "
AN606	" " " " , *ndh*412	-	" " "
AN607	" " " " , *ndh*413	-	" " "

[a] N-methyl-N'-nitro-nitrosoguanidine

TABLE 2-1 Continued

Strain	Relevant Loci	Plasmid	Source or Reference
IY5	*his, ilv, trp, pyr*C, *rps*L	-	This laboratory
IY12	*thi, his, ilv, trp, rps*L, *ndh*406	-	Isogenic P_1 transductant pair derived from IY5 using AN600 as P_1 donor. Young & Wallace (1976).
IY13	" " " " "	-	
IY34	" " " " "	pSF2124	Young *et al.* (1978)
IY35	" " " " "	pIY1	" " " "
IY36	" " " " " , *ndh*406	pIY2	" " " "
IY85	" " " " "	pIY9	I.G. Young & M.I. Poulis, unpublished results

TABLE 2-2 Plasmids

Plasmid	Comments	Source or Reference
pSF2124	Ap^r	So *et al.*, (1975)
pGM706	Ap^r, Tc^r	Hamer & Thomas (1976)
pIY1	pSF2124 with a 1.6Mdal fragment, carrying the *ndh* gene inserted at the *Eco*R1 site	Young *et al.*, (1978)
pIY2	pGM706 with a 4.6Mdal fragment, carrying the *dld* gene, inserted at the *Hin*dIII site	" " "
pIY9	Constructed from pIY1 and carrying an ~0.1Mdal *Eco*R1 fragment, containing 2 copies of the *lac* promoter, adjacent to the *ndh* gene	I.G. Young & M.I. Poulis, unpublished results
pLJ3	pMB9 with an ~0.1Mdal fragment, containing 2 copies of the *lac* promoter, cloned into the *Eco*R1 site	Johnsrud (1978)

(400 rpm), to Klett 200 then harvested using a Sharples continuous flow centrifuge. The cells were then washed (Wallace & Young, 1977), frozen in liquid N_2, and stored at -20^{o}. Contamination of cultures and possible reversion of IY12 were checked by streaking cultures onto brain-heart infusion and mannitol-minimal plates.

Cells were thawed, resuspended in STM buffer (0.25M sucrose, 0.1M N-tris(hydroxymethyl)methyl-2-aminoethane-sulphonic acid, 0.02M magnesium acetate, pH 7.5) at a concentration of 1g wet weight per 3mL buffer, then filtered through gauze. The cells were disrupted using a Ribi cell fractionator at 20,000lb in^{-2}. Cell debris was removed by centrifugation at 15,000 rpm for 1h in a Sorvall SS-34 rotor. The supernatant was centrifuged for 2h at 60,000 rpm in a Spinco 60Ti rotor. The pellet, containing crude membrane particles, was resuspended in STM buffer to a protein concentration of 35 to 45mg mL^{-1} and then washed in two volumes of the same buffer. The washed membrane particles were collected by centrifugation for 2h at 60,000 rpm in a Spinco 60Ti rotor, and resuspended in STM buffer to a protein concentration of approximately 35mg mL^{-1}. Membrane particles were frozen in liquid N_2 and stored at -15^{o}.

Solubilization of Membranes and Hydroxylapatite Column Chromatography

All operations were performed at 0 to 4^{o}.

Membrane particles (12.5mL) were washed and resuspended as described above, then 1.9mL of 20% (w/v) potassium cholate, and 0.93g of solid KCl were added to give the final concentrations of 3% (w/v) cholate and 1M KCl.

The material was mixed thoroughly by inversion, and insoluble material pelleted by centrifugation for 3h at 48,000 rpm in a Spinco SW56 rotor. The clear golden supernatant was loaded onto a column of hydroxylapatite (1.6 x 12cm) equilibrated with not less than 200mL of 5mM potassium phosphate buffer, pH7.5, containing 0.1% (w/v) cholate and 20μM FAD. A 400mL linear gradient, 0.005-1M potassium phosphate, pH 7.5, containing 0.1% (w/v) cholate and 20μM FAD, was applied immediately at a flow rate of approximately 30mL h^{-1}. 4mL fractions were collected. The NADH:ubiquinone oxidoreductase peak, which elutes towards the end of the gradient, was pooled, frozen in liquid N_2 and stored at -15^{o}. A Pharmacia K16/70 column was used for these experiments: the fine polyamide filter, usually in contact with the bed, was replaced by a wad of glass wool, since its use was found to be incompatible with hydroxylapatite at the high phosphate and cholate concentrations used.

Enzyme Assays

Procedures for the determination of oxidase rates have been described previously (Young *et al.*, 1978).

NADH:ubiquinone oxidoreductase activity was measured at 30^{o} by following the ubiquinone-1 dependent NADH oxidation at 340nm in a 1mL reaction mixture containing 50mM TES buffer, pH7.5, 250μM NADH, 40μM FAD, enzyme and 50μM ubiquinone-1. The reaction was started by the addition of ubiquinone-1. Rates were calculated using $\Delta\varepsilon$=6,810M^{-1} cm^{-1} (Schatz & Racker, 1966). Ubiquinone-1 was added as a 1mM solution prepared by diluting ~20mM ethanolic stock

solutions with water, giving a final ethanol concentration in the assay of 0.25% (v/v). Stock ubiquinone-1 solutions were standardized spectrophotometrically as described (Crane & Barr, 1971).

NADH dehydrogenase rates were measured at 30°, and at 420nm, with $K_3Fe(CN)_6$ as electron acceptor, using $\Delta\varepsilon$=1,000M^{-1} cm^{-1}. The 1mL reaction mixture contained 50mM TES buffer, pH 7.5, 250µM NADH, 40µM FAD, enzyme and 1mM $K_3Fe(CN)_6$. The reaction was started by the addition of electron acceptor.

When assaying membrane particles for either activity, 3mM KCN was included in the assay. For convenience, the unit of activity for both reactions is defined as one µmole NADH oxidized min^{-1}.

Preparation of Hydroxylapatite

Hydroxylapatite was made from brushite, essentially as described by Tiselius *et al.* (1956). Special care was taken to avoid overheating and excessive shearing during the conversion. The material appeared as large star-shaped crystals under the microscope with small plate-like 'fines'. The results of calcium and phosphorus analysis of this material were in general agreement with the results obtained by Tiselius *et al.* (1956) and showed less calcium on a molar basis than predicted from the formula $Ca_5(PO_4)_3OH$ (Ca/P=1.444 instead of 1.667). The chromatographic properties of the hydroxylapatite preparations were reproducible in over three dozen enzyme purifications, and were comparable to those of commercial preparations (Bio Rad). Each hydroxylapatite column was used only once.

SDS-polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed on vertical slab gels (10 x 14cm) of 10% or 15% polyacrylamide with a stacking gel of 4.5% polyacrylamide, using an apparatus similar to that described by Studier (1973). The discontinuous buffer system of Laemmli (1970) was used. The preparation of samples and the staining procedure have been described (Young *et al.*, 1978).

Gels were scanned using a Schoeffel model SD3000 spectrodensitometer at 540nm and using a slit width of 0.5mm.

Protein Estimation

Protein concentrations were determined by the method of Lowry *et al.* (1951) using defatted bovine serum albumin (BSA) as standard. BSA stock solutions, nominally 10mg mL^{-1}, were standardized spectrophotometrically using $A^{1\%}_{1cm,280nm}$ = 6.7.

RESULTS AND DISCUSSION

Respiratory Activities in *ndh* Mutant and Control Strains

In assaying the ubiquinone reductase activitity of the NADH dehydrogenase complex, use was made of the short-chain isoprenologue ubiquinone-1 as substrate since the natural electron acceptor ubiquinone-8 has too low a solubility in water to use as a substrate in aqueous enzyme assays. This analogue has been shown to interact normally at the active site of quinone reduction in both membrane and solubilized preparations of the mitochondrial enzyme (Hatefi *et al.*, 1962). Furthermore, the reduction of ubiquinone-1 can support proton gradient formation in

vesicles reconstituted from complex 1 and phospholipids (Ragan & Hinkle, 1975).

NADH dehydrogenase activity of membranes and solubilized preparations was measured with potassum ferricyanide as electron acceptor since, in general, the traces are less curved than with 2,6-dichlorophenolindophenol (DCIP), the other dye commonly used as an electron acceptor. Potassium ferricyanide has been extensively used to assay the NADH dehydrogenase in mitochondria, and has been assumed to accept electrons at a site close to the quinone active site (Singer & Gutman, 1971).

The levels of NADH dehydrogenase activity in membranes prepared from the *ndh* mutant strain, IY12, are normal (Table 2-3). There is however a significant decrease in the level of NADH dependent ubiquinone-1 reduction relative to the wild-type strain, IY13: the ubiquinone reductase activity is 2 to 3%, and the NADH oxidase activity less than 2%, that of the control strain. It has previously been reported (Young & Wallace, 1976) that the DCIP reductase activity in *ndh* mutant strains is 28% that of wild-type strains. The reasons for the discrepancy in the rates of NADH oxidase and NADH:ubiquinone oxidoreductase on the one hand, and the NADH dehydrogenase rate measured with various artificial electron acceptors on the other, will become apparent later.

Cyanide Sensitivity of the Residual NADH oxidase in *ndh* Mutant Membrane Particles

In order to test whether the low NADH oxidase and NADH:ubiquinone oxidoreductase activity seen in *ndh* mutant membrane particles is due to residual activity associated

TABLE 2-3 Respiratory Activities of Membrane Particles Prepared from *ndh* Mutant (IY12) and Wild-Type (IY13) Strains

Activity[a]	IY12	IY13
NADH oxidase	<0.01	0.41
Succinate oxidase	0.15	0.15
D-lactate oxidase	0.37	0.21
NADH:ubiquinone oxidoreductase	0.02	0.74
NADH:$K_3Fe(CN)_6$ oxidoreductase	0.23	0.32

[a] Oxidase rates are expressed as µg atoms O consumed min^{-1} mg^{-1} protein and dehydrogenase rates as µmoles NADH oxidized min^{-1} mg^{-1} protein.

with the respiratory NADH dehydrogenase complex, the cyanide sensitivity of the NADH oxidase activity was examined.

The wild-type NADH oxidase is almost completely inhibited by 3mM KCN. However, the low NADH oxidase activity of *ndh* mutant membrane particles is not affected by KCN even at concentrations as high as 20mM (Figure 2-1), suggesting that it is unrelated to the respiratory activity. AN602 is a leaky *ndh* mutant, having 10 to 15% of the wild-type level of NADH oxidase in the cytoplasmic membrane (I.G. Young, unpublished results). Interestingly, the NADH oxidase in membrane particles prepared from this strain is inhibited approximately 37% by 3mM KCN (Figure 2-1), suggesting that the respiratory NADH dehydrogenase is partially active in this strain.

From Figure 2-1 it can be seen that the *ndh* mutant strain AN606 is totally deficient in the cyanide sensitive NADH oxidase and therefore the possibility can be excluded that there exists a second NADH dehydrogenase under these growth conditions linked to the respiratory chain.

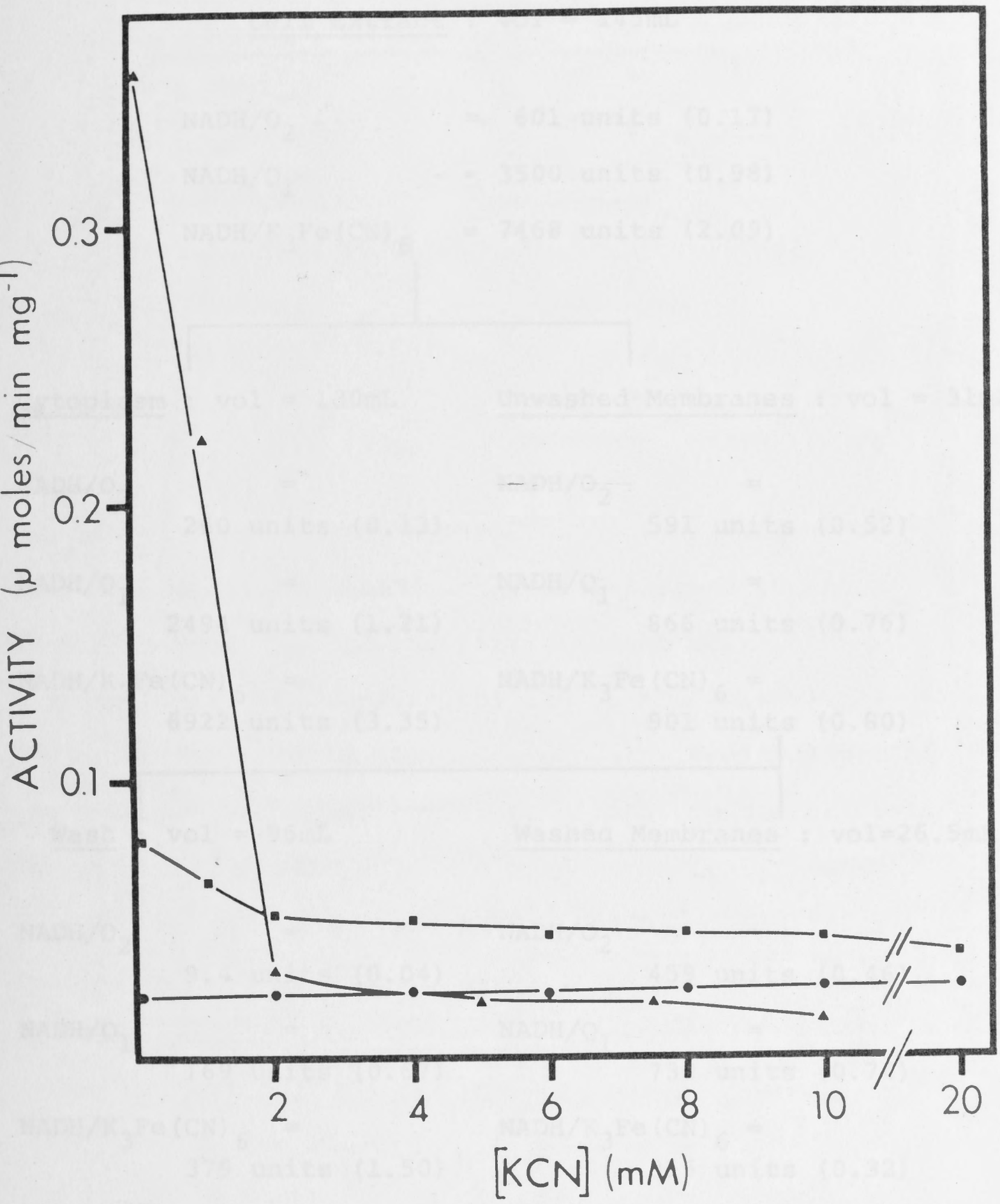
The nature of the cyanide insensitive NADH oxidase is not clear, but it may be related to the cytochrome-independent NADH oxidase reported by Haddock & Schairer (1973).

Solubilization and Chromatography of NADH:ubiquinone Oxidoreductase from Wild-Type Membrane Particles

Initial attempts at purifying the respiratory NADH dehydrogenase complex were carried out with membrane particles prepared from IY13.

As can be seen in Figure 2-2, there is a high NADH

FIGURE 2-1. Inhibition of NADH Oxidase Activity in Membrane Particles Prepared from JP2140, AN602 and AN606.



Membrane particles were prepared from strains JP2140, AN602 and AN606 as described in the text, and assayed for NADH oxidase activity spectrophotometrically (see Chapter 4) in the presence of varying concentrations of KCN.

▲——▲ JP2140; ■——■ AN602; ●——● AN606.

FIGURE 2-2. Distribution of NADH Oxidase and NADH Dehydrogenase Activities During Preparation and Solubilization of Membrane Particles from IY13

Cell Extract : vol = 145mL

$NADH/O_2$ = 601 units (0.17)

$NADH/Q_1$ = 3500 units (0.98)

$NADH/K_3Fe(CN)_6$ = 7468 units (2.09)

Cytoplasm : vol = 130mL

$NADH/O_2$ = 260 units (0.13)

$NADH/Q_1$ = 2494 units (1.21)

$NADH/K_3Fe(CN)_6$ = 6922 units (3.35)

Unwashed Membranes : vol = 31mL

$NADH/O_2$ = 591 units (0.52)

$NADH/Q_1$ = 866 units (0.76)

$NADH/K_3Fe(CN)_6$ = 901 units (0.80)

Wash : vol = 96mL

$NADH/O_2$ = 9.4 units (0.04)

$NADH/Q_1$ = 169 units (0.67)

$NADH/K_3Fe(CN)_6$ = 379 units (1.50)

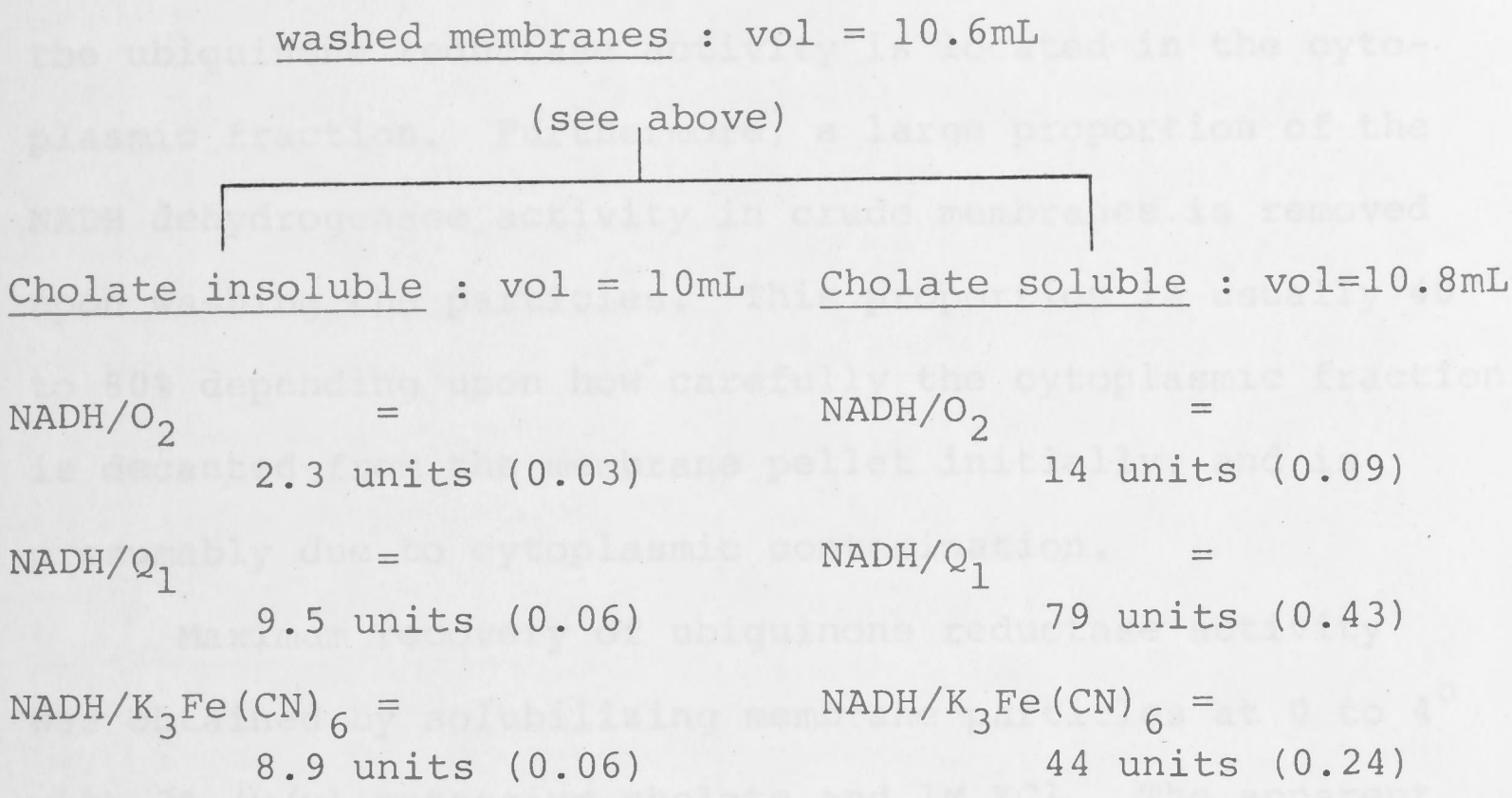
Washed Membranes : vol=26.5mL

$NADH/O_2$ = 459 units (0.46)

$NADH/Q_1$ = 733 units (0.74)

$NADH/K_3Fe(CN)_6$ = 315 units (0.32)

FIGURE 2-2 Continued.



90L of IY13 were grown on mannitol-minimal medium plus casamino acids, and membrane particles prepared and washed as described in the text: 10.6mL of washed membrane particles were solubilized with 3% (w/v) potassium cholate, 1M KCl. The cholate-insoluble pellet was resuspended in STM buffer to a final volume of 10mL. Abbreviations: $NADH/O_2$, NADH oxidase; $NADH/Q_1$, NADH: ubiquinone oxidoreductase; $NADH/K_3Fe(CN)_6$, NADH:ferricyanide oxidoreductase. Figures in parentheses refer to specific activities.

dehydrogenase activity in the cytoplasm of *E. coli*. Approximately 88% of the ferricyanide reductase and 74% of the ubiquinone reductase activity is located in the cytoplasmic fraction. Furthermore, a large proportion of the NADH dehydrogenase activity in crude membranes is removed upon washing the particles. This proportion is usually 40 to 80% depending upon how carefully the cytoplasmic fraction is decanted from the membrane pellet initially, and is presumably due to cytoplasmic contamination.

Maximum recovery of ubiquinone reductase activity was obtained by solubilizing membrane particles at 0 to 4^{o} with 3% (w/v) potassium cholate and 1M KCl. The apparent recovery of activity was 20 to 25% with a slight decrease in specific activity (Table 2-4). This drop in specific activity is probably due in part to the reversible inhibition of NADH:ubiquinone oxidoreductase activity by the high concentration of cholate in the sample, as the experiments illustrated in Figures 2-3 and 2-4 suggest.

When the solubilized material is chromatographed on hydroxylapatite, several peaks of NADH dehydrogenase activity are resolved, but there is only one major peak of NADH: ubiquinone oxidoreductase (Figure 2-5(a)). The major peak of ubiquinone reductase activity was identified as the respiratory NADH dehydrogenase since it is absent from the chromatogram of an identical column where solubilized IY12 membrane particles were chromatographed (Figure 2-5(b)). The NADH:ubiquinone oxidoreductase has no measurable activity with NADPH as electron donor (as expected for the respiratory enzyme).

The other NADH dehydrogenase activities are present

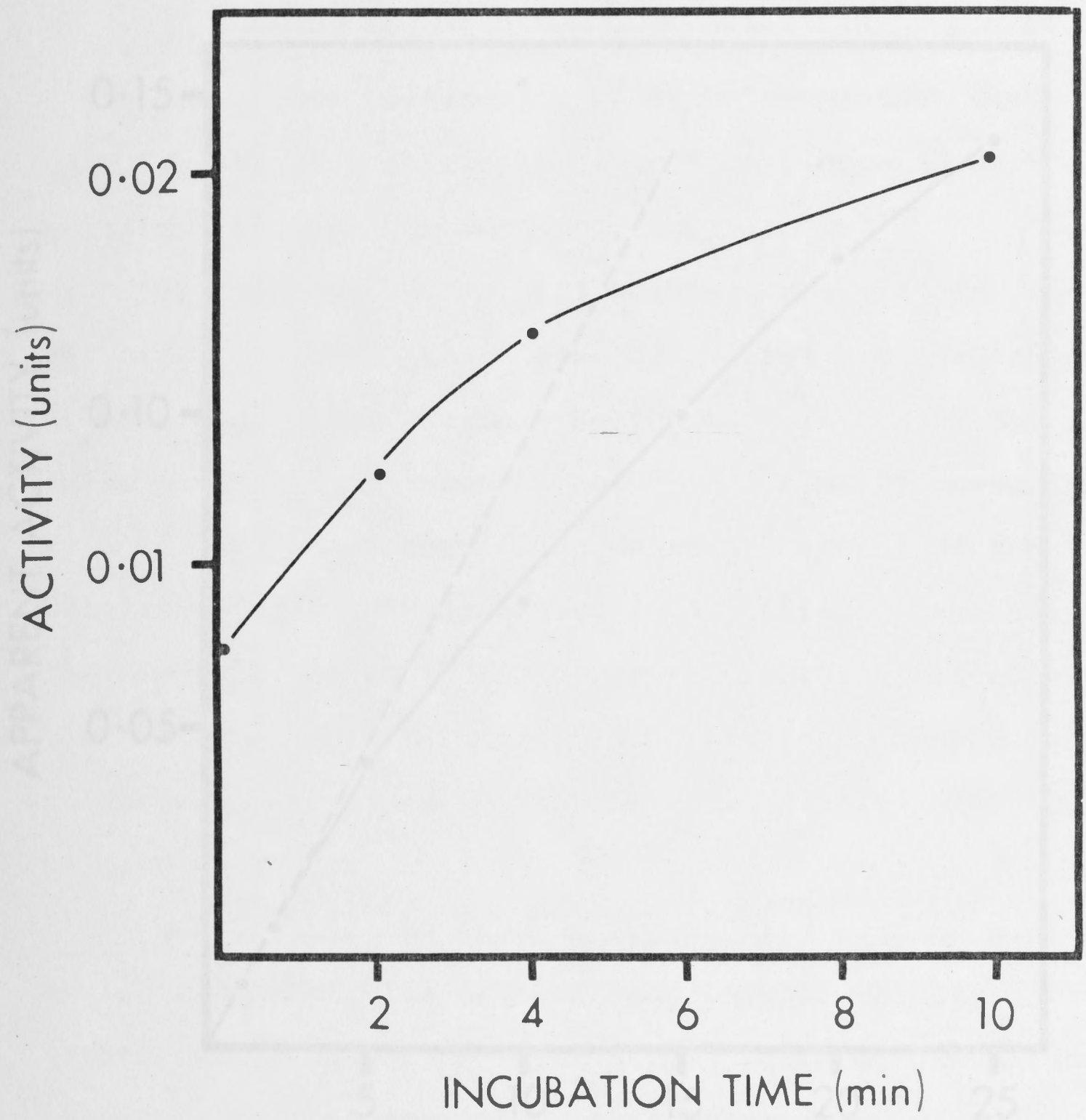
TABLE 2-4 Purification of NADH:ubiquinone Oxidoreductase from IY13 Membrane Particles[a]

	Volume (mL)	Activity[b] (Units mL^{-1})	(Units)	Protein ($mg\ mL^{-1}$)	(mg)	Specific Activity (Units mg^{-1})	Yield (%)	Purification (fold)
Crude Membranes	12.5	25.5	319	36.5	456	0.70	(100)	(1)
Washed Membranes	10.6	25.2	268	37.2	394	0.68	86	0.97
Cholate-Soluble	10.2	7.06	72.0	17.7	180	0.40	23	0.57
Hydroxylapatite	79	2.09	165	0.111	8.8	18.8	52	27

[a] For details see Experimental section.

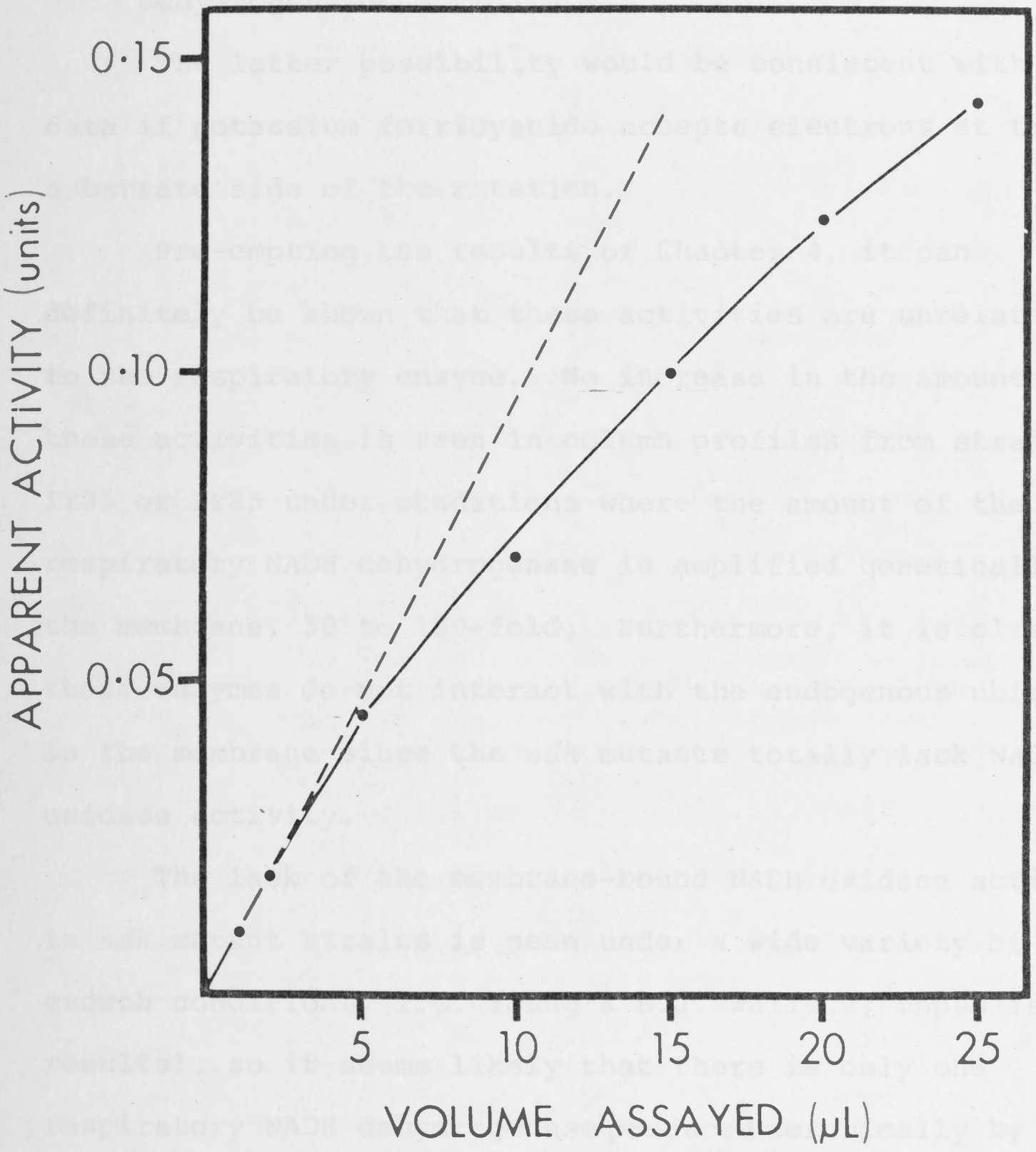
[b] 1 unit ≡ 1 μmole NADH oxidized min^{-1} mg^{-1}

FIGURE 2-3. Relief of Inhibition of Cholate-Solubilized Membrane Particles by Preincubation in Assay Mix.



IY13 membrane particles were solubilized with potassium cholate as described in the text: 2µL of cholate-soluble material was preincubated for various lengths of time, at 30°, in 1mL of 50mM TES buffer, pH7.5, 40µM FAD, 250µM NADH and 3mM KCN. The reaction was started by the addition of 50µL ubiquinone-1 solution (1mM). Preincubation under identical conditions did not enhance the activity of either membrane particles or pure NADH:ubiquinone oxidoreductase.

FIGURE 2-4. NADH:ubiquinone Oxidoreductase Activity of Cholate-Solubilized Particles as a Function of Added Enzyme.



For details of solubilization and assay procedures see text.

in the IY12 profile. This can be interpreted in two ways:

(1) These activities are unrelated to the respiratory NADH dehydrogenase.

(2) They are fragments or different solubilized forms of the NADH dehydrogenase complex, containing the primary dehydrogenase.

The latter possibility would be consistent with the data if potassium ferricyanide accepts electrons at the substrate side of the mutation.

Pre-empting the results of Chapter 4, it can definitely be shown that these activities are unrelated to the respiratory enzyme. No increase in the amounts of these activities is seen in column profiles from strains IY35 or IY85 under conditions where the amount of the respiratory NADH dehydrogenase is amplified genetically, in the membrane, 50 to 100-fold. Furthermore, it is clear that these enzymes do not interact with the endogenous ubiquinone in the membrane since the *ndh* mutants totally lack NADH oxidase activity.

The lack of the membrane-bound NADH oxidase activity in *ndh* mutant strains is seen under a wide variety of growth conditions (I.G. Young & B.J. Wallace, unpublished results), so it seems likely that there is only one respiratory NADH dehydrogenase produced aerobically by *E. coli*, and which is defective in *ndh* mutant strains. Estimations of the cytochrome and quinone content of IY12 (see Chapter 3) reveal that the lack of the respiratory NADH oxidase in this strain is not due to a defect in the biosynthesis of ubiquinone or any of the detectable cytochromes, and is therefore due specifically to an inactive NADH dehydro-

genase complex.

There are contaminating NADH dehydrogenase activities in the membrane as well as in the cytoplasm. The cytoplasmic activity could be due to enzymes such as lipoamide dehydrogenase (Dancey *et al.*, 1976), but the nature of the membrane-bound activities is not clear. Owen and Kaback (1979) have demonstrated the presence of two or possibly three NADH dehydrogenases in the plasma membrane of *E. coli* by crossed immunoelectrophoresis of spheroplasts prepared by osmotic shock, and Dancey *et al.* (1976) have concluded that there is more than one NADH dehydrogenase, in experiments on the inhibition of NADH:DCIP oxidoreductase by 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) in similar spheroplast preparations.

What is now clear is that the NADH dehydrogenase activity measured in membrane preparations is due to the combined activity of several enzymes, and the levels of activity with various electron acceptors in the *ndh* mutant strain, compared to the wild-type strain, reflect the relative contribution of the respiratory enzyme to the overall activity. It is not valid to relate the contributions to the membrane-bound activity of any particular enzyme to its relative amount seen on the hydroxylapatite column chromatogram, since conditions were chosen specifically to maximize the recovery of the respiratory enzyme. However it is clear from the data presented in Table 2-3 and from the previous results (Young & Wallace, 1976) that nearly all of the activity with ubiquinone-1 and the majority of the activity with DCIP seen in membrane preparations is due to the respiratory enzyme, whereas most of the membrane-bound

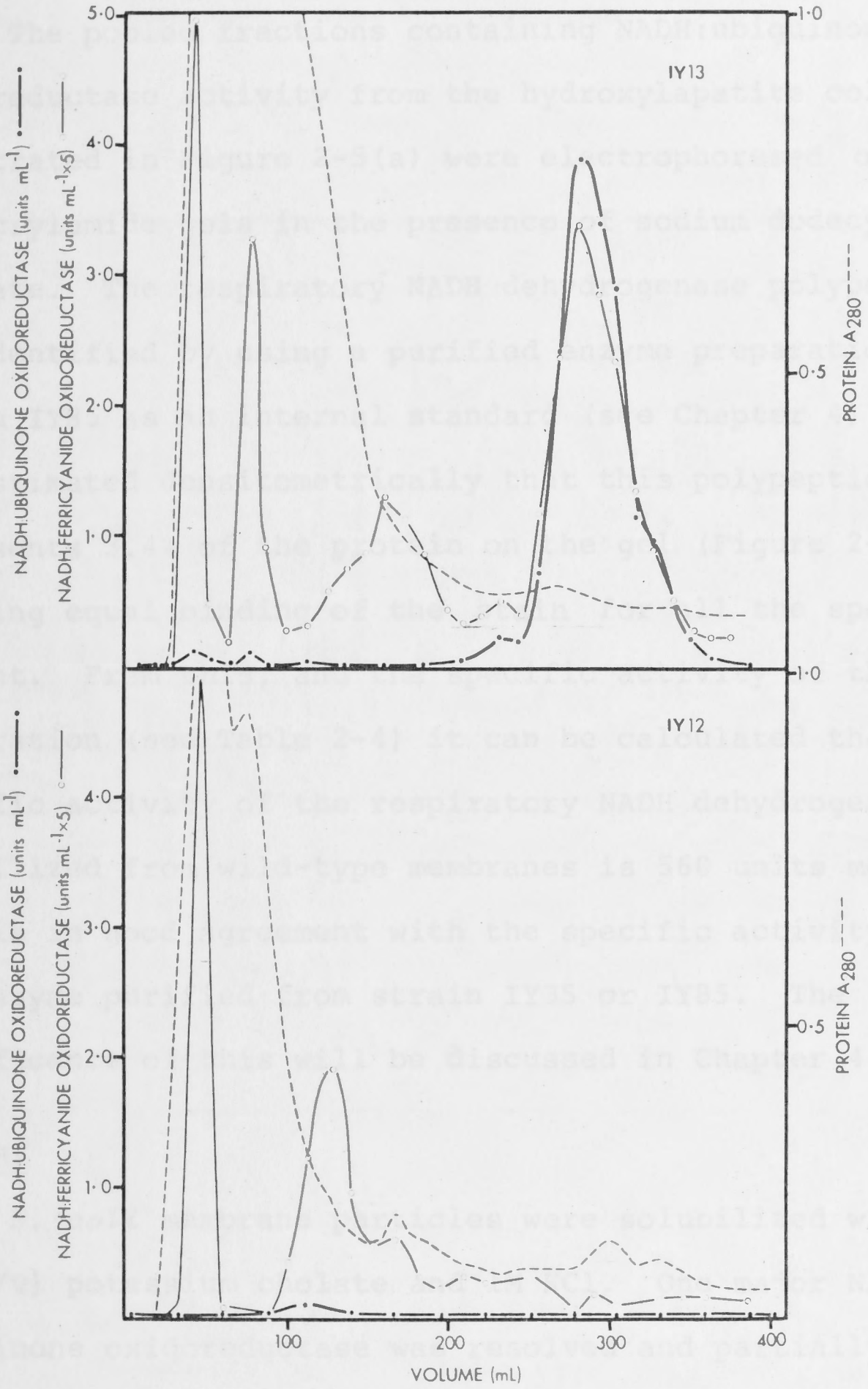
ferricyanide reductase activity is due to the contaminating (in the sense of non-respiratory-chain-linked) activities.

Attempted Purification of NADH:ubiquinone Oxidoreductase from Strain IY13

Conditions for the chromatography of the NADH:ubiquinone oxidoreductase on hydroxylapatite were optimized to give the conditions described in Figure 2-5. The chromatographic behaviour of the enzyme was found to depend upon such parameters as the concentration of cholate and FAD present in the column and gradient buffers, the steepness of the phosphate gradient and the amount of solubilized membranes chromatographed, relative to the column bed volume. Under optimum conditions, excellent separation of activity from the bulk of the protein is achieved, owing to the unusually tight binding of the enzyme complex to hydroxylapatite. This may reflect phospholipid binding to the phosphate sites in the hydroxylapatite crystal lattice rather than protein-hydroxylapatite interactions since such tight binding is also a characteristic of phosphoproteins. Using this single column step a purification of approximately 30-fold over the activity in membrane particles can be achieved (Table 2-4).

It was not possible to obtain highly purified preparations of the NADH:ubiquinone oxidoreductase from IY13. As will become apparent (see Chapter 4), the enzyme is present in very small quantities in wild-type membranes. The ubiquinone reductase activity proved to be very labile, and all attempts at further purification led to large losses in activity. Amongst the approaches tested were rechromatography on hydroxylapatite under different

FIGURE 2-5. Chromatography of Cholate-Solubilized Membrane Particles on Hydroxylapatite.



See Experimental section for details. A_{280} was monitored continuously with an Isco model UA-5 absorbance monitor. Column flow-rate (30mL h^{-1}) was kept constant by using a Pharmacia P-3 peristaltic pump. 4mL fractions were collected.

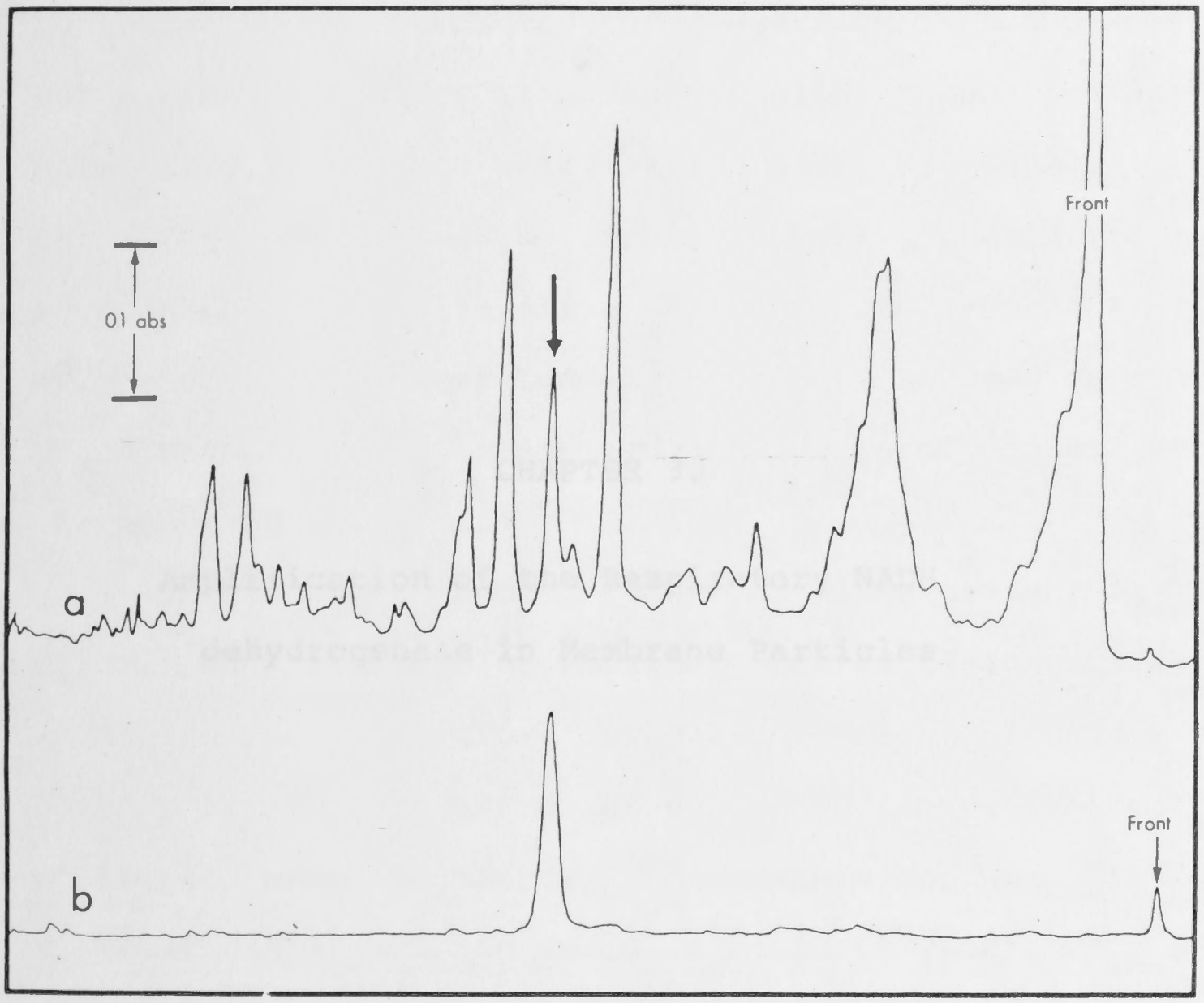
conditions, gel permeation and affinity chromatography (the latter using a variety of ligands).

The pooled fractions containing NADH:ubiquinone oxidoreductase activity from the hydroxylapatite column illustrated in Figure 2-5(a) were electrophoresed on 10% polyacrylamide gels in the presence of sodium dodecyl sulphate. The respiratory NADH dehydrogenase polypeptide was identified by using a purified enzyme preparation from strain IY85 as an internal standard (see Chapter 4). It was estimated densitometrically that this polypeptide represents 3.4% of the protein on the gel (Figure 2-6), assuming equal binding of the stain for all the species present. From this, and the specific activity of the preparation (see Table 2-4) it can be calculated that the specific activity of the respiratory NADH dehydrogenase solubilized from wild-type membranes is 560 units mg^{-1}. This is in good agreement with the specific activity of the enzyme purified from strain IY35 or IY85. The significance of this will be discussed in Chapter 4.

SUMMARY

E. coli membrane particles were solubilized with 3% (w/v) potassium cholate and 1M KCl. One major NADH: ubiquinone oxidoreductase was resolved and partially purified by hydroxylapatite chromatography of the solubilized material. This enzyme has been identified as the respiratory NADH dehydrogenase since it is absent from chromatograms of solubilized material from the *ndh* mutant IY12. Several other NADH dehydrogenases are present in *E. coli* membranes: comparative studies with strains IY12 and IY13 have shown that they are apparently unrelated to the respiratory enzyme.

FIGURE 2-6. SDS Polyacrylamide Gel Electrophoresis of NADH:ubiquinone Oxidoreductase Partially Purified from IY13.



The NADH:ubiquinone oxidoreductase peak, from hydroxylapatite chromatography of solubilized IY13 membrane particles (fractions 64 to 85, see Figure 2-5(a)), was pooled, electrophoresed on 10% SDS polyacrylamide gels and scanned densitometrically (top). The NADH dehydrogenase flavoprotein (marked with arrow) was identified using pure enzyme prepared from strain IY85 (bottom trace; see Chapter 4) and was calculated to be 3.4% of the protein in the sample. From the specific activity of the pooled preparation, 18.9 units mg^{-1} (Table 2-4), the specific activity of the pure enzyme from wild-type cells is estimated to be 560 units mg^{-1} assuming one active site per 45,000 polypeptide chain.

CHAPTER 3.

Amplification of the Respiratory NADH dehydrogenase in Membrane Particles

INTRODUCTION

Preliminary experiments on the purification of the respiratory NADH dehydrogenase complex (see Chapter 2) indicated that the enzyme would be difficult to purify by conventional techniques, and suggested that a new approach was necessary. Since it is labile after solubilization and refractory to further purification after hydroxylapatite chromatography, it was desirable to purify the enzyme as much as possible while still in the membrane-bound state. Recent advances in genetic engineering suggested approaches which might be used to amplify the levels of the enzyme in the membrane *in vivo*.

The extrachromosomal factor ColE1 is present in *E. coli* under relaxed control, at a level of approximately 24 copies per cell (Clewell, 1972). Several plasmid vectors have been constructed which are derivatives of ColE1, and exist in the cell in multiple copies. These are suitable as cloning vehicles since they possess a single site for given restriction endonucleases and carry suitable antibiotic resistance markers for ease of selection.

When a gene is cloned into such plasmids, the amounts of enzyme coded by that particular gene can be elevated to a level consistent with the increased gene copy-number in the cell (Hershfield *et al.*, 1974; Vapnek *et al.*, 1976; Wickner *et al.*, 1976; Raetz *et al.*, 1977; Steffen & Schleif, 1977).

Cloning a particular gene into multicopy plasmids is conveniently carried out if there already exists an enriched source of that gene, *e.g.* if it is carried on a transducing phage (Hershfield *et al.*, 1974; Steffen & Schleif, 1977) or

F factor (Vapnek *et al.*, 1976). Where this is no enriched source, the gene must be isolated from total chromosomal DNA. Several techniques are available to do this. Clarke and Carbon (1976) established a colony bank of over 2,000 clones by shearing *E. coli* DNA and ligating the fragments to ColE1 using the poly (dA.dT) connector method (Jackson *et al.*, 1972; Lobban & Kaiser, 1973). Collins *et al.* (1976) and Koslov *et al.* (1977) digested chromosomal DNA with the restriction endonuclease *Eco*RI and ligated the fragments with plasmid DNA digested with the same enzyme. The resulting hybrid plasmids were then transformed directly into relevant mutant strains. A similar approach was used by Borck *et al.* (1976) to prepare specialized lambda transducing phages *in vitro*.

Up to this time only genes coding for soluble cytoplasmic enzymes had been successfully cloned. It remained to be seen whether amplification of enzyme levels could be achieved through the cloning of genes coding for membrane-bound enzymes, or whether special requirements, such as for insertion into the membrane or assembly into respiratory complexes, would circumvent this.

The gene coding for the respiratory NADH dehydrogenase was cloned by a method similar to that used by Collins *et al.* (1976) and Koslov *et al.* (1977) (Young *et al.*, 1978). Total chromosomal DNA was digested separately with three different restriction endonucleases, *Eco*RI, *Bam*I and *Hin*dIII, to increase the chances of cloning the gene intact. A gene cannot be successfully cloned with a particular endonuclease if it contains a restriction site for that enzyme. There is less chance that the gene will contain a restriction site for

each of the three enzymes used. Cloning vectors which possess a single site for a given restriction endonuclease were then digested with that enzyme and ligated with the corresponding pool of DNA: thus pGM706, which possesses a single *Hin*dIII site (Hamer & Thomas, 1976), was digested with *Hin*dIII and ligated with the fragments of DNA obtained from *Hin*dIII digested chromosomal DNA, to produce a chromosomal library. Similar libraries were constructed from *Bam*I and *Eco*RI digested chromosomal DNA and the plasmid pSF2124, which possesses a single site for each of these two restriction endonucleases (So *et al.*, 1975).

In theory, the hybrid plasmids so produced can be transformed directly into *ndh* point mutants and selection made for those cells which have received plasmids (using suitable antibiotics) and for complementation of the mutant phenotype (in this case using growth on mannitol as one's criterion). Unfortunately it was found that the *ndh* mutant strains used as recipients are poor transformants. To get over this problem, use was made of the fact that the vectors used, pSF2124 and pGM706, though non-transmissible in themselves, can be mobilized by factors such as F (Young & Poulis, 1978). The hybrid plasmids were therefore transformed into an efficient transforming strain, C600, then transferred to the *ndh* mutant recipient strain, IY12, by F-mediated conjugation.

Two hybrid plasmids were obtained, one from the *Eco*RI pool and the other from the *Hin*dIII pool, which complement the *ndh* phenotype. These plasmids were designated pIY1 and pIY2 respectively.

Once isolated, the plasmids were transformed directly into *ndh* mutant recipients, and were shown to complement all the *ndh* mutants so far isolated (I.G. Young, unpublished results). It has been shown (Young *et al.*, 1978) that pIY1 and pIY2 contain a 1.6 and a 4.6Mdalton cloned DNA insert respectively.

Since the former hybrid plasmid contains the smaller insert, it was selected for further study. In this chapter, the use of pIY1 in amplifying the level of NADH: ubiquinone oxidoreductase in the membrane will be described. Furthermore it will be shown that pIY2 does not carry the *ndh* gene, and complements the *ndh* mutant phenotype by a totally different mechanism to pIY1. Respiratory activities of both hybrid plasmid-containing strains are presented, and compared to those of *ndh* mutant and wild-type strains.

EXPERIMENTAL

Media and Growth of Bacteria

For details of the media used, and the growth of 10L cultures in glass fermenters, see Experimental section, Chapter 2.

In growing cultures of strains harbouring plasmids carrying drug resistance determinants, the following precautions were employed: rubber hoses were attached to the air outlets of the fermenter vessels, and the air trapped in glass flasks stoppered with cotton wool, and containing 1% (v/v) hibitane^{R}. The effluent obtained on harvesting the cells was collected in 25L Nalgene^{R} bottles, and autoclaved prior to disposal, as was all material coming in contact with the cultures.

Agar plates used to prepare inocula of IY35 and IY36 contained 2% (w/v) agar and 25μg mL^{-1} ampicillin in mannitol-minimal medium, and were stored at 4^{o} and used within 4 to 6 weeks of preparation. Cultures of IY35 and IY36 were also checked after growth, on the above plates, for retention of plasmid: this medium tests for both the presence of the ampicillin resistance gene on the cloning vector and complementation of the *ndh* phenotype by the hybrid plasmid.

Growth Tests

Growth tests were done, throughout, in 125mL flasks fitted with a side-arm which allows the turbidity of the culture to be measured directly, using a Klett-Summerson colorimeter.

For the measurement of growth rates, flasks were shaken (200 rpm) in a water bath at 37^{o}. The flasks were cleaned alternately in alcoholic KOH and chromic acid before being rinsed thoroughly in distilled water. The medium used and the concentrations of the supplements are described in Chapter 2. Glucose and mannitol were added at a final concentration of 30mM. Succinate was added at a final concentration of 4mM and prepared, for growth tests, from succinic acid, rather than the sodium salt, and adjusted to pH 7.0 with NaOH. Casamino acids (Difco, vitamin free) were added at a final concentration of 0.1% (w/v).

For the determination of growth in limiting concentrations of isoleucine plus valine, cultures of IY35 were grown in 10mL mannitol-minimal medium except that the levels of isoleucine plus valine were at the indicated final concentrations.

Amino Acid Starvation Experiments with IY35

3 x 10L cultures of IY35 were grown, as described above, in mannitol-minimal medium, except that the final concentrations of isoleucine and valine were 0.1mM each. Growth ceased abruptly when the culture turbidity reached Klett 100. The cultures were incubated with aeration (12L min^{-1}) and stirring (400 rpm) for 1, 2 and 4h respectively, after which time growth was restarted by the addition of 0.3mM isoleucine and 0.3mM valine to each culture. Cells were harvested as described above, when the culture turbidity reached Klett 200.

Recovery of Growth after Chloramphenicol Inhibition

To determine the recovery of growth after inhibition with chloramphenicol, 2 x 50mL cultures of IY35 were grown in mannitol-minimal medium to Klett 135. Chloramphenicol was added at this point to a final concentration of 150µg mL^{-1}, which completely inhibited further growth. At time intervals of 0, 1, 2, 4, 6, 8 and 12h, 10mL aliquots were transferred to sterile, capped plastic tubes, centrifuged, and the cells washed once in 10mL of sterile medium minus mannitol and supplements. The washed cells were resuspended in 10mL of mannitol-minimal medium and subsequent growth recorded.

Determination of Optimum Inhibitory Concentration of Chloramphenicol

For the determination of the optimum inhibitory concentration of chloramphenicol, 10mL cultures of IY35 were grown in mannitol-minimal medium containing 25, 50, 75 and 100µg mL^{-1} of the antibiotic, in side arm flasks.

Amplification of IY35 with Chloramphenicol

10L cultures of IY35 were grown, as described above, in mannitol-minimal medium, unless otherwise specified, containing 0.1% (w/v) casamino acids. When a Klett value of 100 was reached, 0.5g chloramphenicol was added and the culture incubated, with aeration (12L min^{-1}) and stirring (400 rpm), for 4h, unless otherwise specified. Cells were then harvested, washed in 300mL of sterile medium and collected by centrifugation for 1h at 9,000 rpm in a Sorvall GS-3 rotor. The washed cells were then resuspended in an equal volume of fresh mannitol-minimal medium containing 0.1% (w/v) casamino acids. The initial turbidity of the culture on reinoculation was generally Klett 80, and growth was continued until the turbidity doubled. The cells were then harvested and membrane particles prepared (see below).

Preparation of Membrane Particles and Assay Procedures

Membrane particles were prepared as described in the Experimental section, Chapter 2, except that the final wash was omitted since it was found that the specific activities of NADH oxidase and NADH:ubiquinone oxidoreductase were not significantly altered by this step.

D-lactate dehydrogenase was assayed by measuring the phenazine methosulphate-coupled reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (Futai, 1973). Duroquinol oxidase was measured polarographically using a Clark-type oxygen electrode. The reaction mixture contained 0.05mL of membrane particles, 0.5mg sodium dithionite and 0.6mg duroquinone (added as an ethanolic solution, concentration 30mg mL^{-1}) in 2.5mL STM buffer (see Chapter 2). The concentration of dissolved oxygen was

taken to be 450ng atoms mL^{-1} (Chappell, 1964).

Details of other assays are presented in Chapter 2.

Quinone Estimations on Whole Cells

Bacteria were grown in 10L batch cultures and harvested at Klett 200. The cells were then washed in 300mL of STM buffer (see Chapter 2) and pelleted by centrifugation for 1h at 8,000 rpm in a Sorvall GS-3 rotor.

10g of cell paste was transferred to an extraction thimble (Whatman, cellulose) and extracted with 150mL boiling acetone for 4h in a Soxhlet apparatus (Young *et al.*, 1973). The acetone was evaporated over steam, and the residue extracted with 3 x 50mL petroleum spirit (b.p. 60-80^{o}). The combined extract was rotary evaporated to a small volume and applied to a silica gel, GF_{254}, thin layer plate (0.5mm thick, 18 x 20cm) as a band approximately 2 x 8cm. The chromatograms were developed in chloroform: petroleum spirit (b.p. 60-80^{o}), 7:3 (v/v). The dark yellow ubiquinone band, R_f = 0.3, and the faint yellow naphthoquinone band (containing both menaquinone and demethylmenaquinone), R_f = 0.7, were each eluted with 6 x 2mL absolute ethanol.

Ubiquinone was determined spectrophotometrically by measuring the difference in absorbance at 275nm after reduction with solid $NaBH_4$, and using $\Delta\varepsilon$ = 12,700cm^{-1} M^{-1} (Crane & Barr, 1971).

Demethylmenaquinone was determined by measuring the visible absorbance due to the violet chromophore formed between 2-β-alkenyl-1,4-naphthoquinones and ethanolic KOH (Baum & Dolin, 1965): to 0.8mL of the naphthoquinone extract, in a 1mL quartz cuvette, was added 0.2mL of freshly

made 1M KOH, and the absorbance at 560nm followed. Values were calculated from the maximum absorbance change using $\Delta\varepsilon = 8{,}300 cm^{-1} M^{-1}$ (Alexander & Young, 1978b).

The total naphthoquinone content was determined by measuring the difference in optical density at 245nm following the addition of solid $NaBH_4$ to 1mL of the naphthoquinone solution containing 10µL of 1M sodium acetate buffer, pH5.0. The contribution to the absorbance change due to demethylmenaquinone was calculated from its content, estimated as described above, and using $\Delta\varepsilon = 19{,}800 cm^{-1} M^{-1}$ (Dunphy & Brodie, 1971). The menaquinone content was obtained from the difference, assuming $\Delta\varepsilon = 25{,}800 cm^{-1} M^{-1}$ (Dunphy & Brodie, 1971).

Determination of Cytochrome Levels in Membrane Particles

"Dithionite-reduced minus oxidized" difference spectra were recorded at room temperature using an Aminco Chance DW2 dual wavelength spectrophotometer: 0.9mL of membrane particles (protein concentration = 35 to 45mg mL^{-1}) were diluted with 2.1mL STM buffer (see Chapter 2), and 1mL transferred to sample and reference cuvettes. To the reference cuvette was added 2.5µmoles H_2O_2 and 4 units catalase (beef liver, Sigma) and to the sample cuvette was added a few grains of sodium dithionite. The spectrum was recorded at the following instrument settings: full scale deflection, 0.2*A*; spectral bandpass, 1nm; scan-rate, 1nm sec^{-1}; instrument response, medium. The contents of the reference cuvette were then reduced with excess dithionite and the baseline spectrum recorded. The concentrations of cytochromes b_1 and *d* were measured as described previously (Wallace & Young, 1977(b) using the

following molar absorption coefficients and wavelength pairs: cytochrome b_1 17,500cm^{-1} M^{-1} at 560 minus 575nm (Jones & Redfearn, 1966) and cytochrome *d*, 8,500cm^{-1} M^{-1} at 615 minus 630nm (ibid.). For determination of cytochrome *o*, the particles in the sample cuvette were bubbled vigorously with CO for 2min and the 'CO-reduced minus reduced' difference spectrum was recorded using the same instrument settings as above. Cytochrome *o* content was estimated at the wavelength pair 415 minus 430nm using the molar absorption coefficient 80,000cm^{-1} M^{-1} (Chance, 1961).

Low Temperature Cytochrome Spectra

'Dithionite-reduced minus oxidized' difference spectra of membrane particles were obtained at liquid N_2 temperature (77K) using the Aminco Chance DW2 dual wavelength spectrophotometer with the low temperature attachment. Other details are given in the figure legends.

RESULTS AND DISCUSSION

Respiratory Activities in Membrane Particles Prepared from Strain IY35

Membrane particles were prepared from strains IY35 (which carries the hybrid plasmid pIY1), IY34 (which carries the plasmid vector pSF2124) and IY13, and respiratory activities determined for both the membrane and cytoplasmic fractions (Table 3-1).

The results show that there is an approximately seven-fold elevation in the level of NADH:ubiquinone oxidoreductase in the membrane of IY35 compared to the wild-type strain IY13, without appreciable amplification of the activity in the cytoplasm. Comparing the results for

TABLE 3-1 Respiratory Activities of Membrane and Cytoplasmic Fractions from IY35 and Control Strains

Activity[a]	IY13		IY34		IY35	
	M[b]	C[c]	M[b]	C[c]	M[b]	C[c]
NADH oxidase	0.679	0.041	0.684	0.053	2.54	0.170
Succinate oxidase	0.362	ND	0.198	ND	0.498	ND
D-lactate oxidase	0.240	ND	0.289	ND	0.574	ND
NADH:ubiquinone oxidoreductase	0.576	0.718	0.577	0.423	3.86	0.776
NADH:ferricyanide oxidoreductase	1.60	0.950	1.01	1.04	0.790	1.55

[a] Oxidase rates expressed as µg atoms O consumed min^{-1} mg^{-1} protein and dehydrogenase rates as µmoles NADH oxidized min^{-1} mg^{-1} protein.

[b] Membrane fraction.

[c] Cytoplasmic fraction.

Other details given in text.

IY34 and IY35, it is apparent that this is not an effect of the plasmid vector itself but, rather, due to the cloned DNA insert. The results for five membrane preparations (two each of IY35 and IY13, and one of IY34) showed that IY35 has a 7 to 10-fold amplification of NADH:ubiquinone oxidoreductase levels relative to the control strains (average 8.8-fold).

The amount of NADH:ubiquinone oxidoreductase in IY35 membranes is consistent with that expected if the level is proportional to the gene copy-number in the cell, and is of the same magnitude as the amplification of the various soluble enzymes whose genes have been cloned into multicopy plasmids to date (Hershfield *et al.*, 1974; Vapnek *et al.*, 1976; Wickner *et al.*, 1976; Raetz *et al.*, 1977; Steffen & Schleif, 1977). Although the number of pIY1 molecules per cell has not been determined, an idea of the expected levels can be gained from the copy-number of pSF2124 itself, which has been estimated to be approximately 20 copies per cell (So *et al.*, 1975). The degree of amplification expected is correctly related to the gene copy-number per chromosome-equivalent. The exact number of chromosome molecules per cell depends upon the stage of growth of the culture as well as the growth conditions used, but can vary between 2 (Cooper & Helmstetter, 1968) and 4 (Clewell, 1972). On this basis we would expect an amplification of between 5 and 10-fold over the wild-type levels of a particular protein if the gene coding for that protein were cloned into pSF2124, and there were no other

controls on gene expression*.

Amplification of Plasmid Copy-Number

An important property of ColE1, as well as its derivative plasmids such as pSF2124, is that it can continue to replicate when cellular protein synthesis is inhibited. Replication of chromosomal DNA and cell division cease under such conditions, resulting in a very high plasmid copy-number (Clewell, 1972). Experiments were undertaken to try to improve enzyme levels by making use of this property to further increase the gene copy-number in the cell.

The logic behind the amplification experiments can be summarised as follows: cultures of IY35 were grown to one half the cell density desired for harvesting, then protein synthesis was inhibited either by amino acid starvation or by the use of specific inhibitors. The cultures were incubated under such conditions for varying lengths of time to allow the gene copy-number to increase, then the inhibition was relieved and the cells grown for one generation to allow expression of the amplified genes.

Amplification of Enzyme Levels by the Use of Amino Acid Starvation

An effective way to inhibit protein synthesis in an

---

*In actual fact, this approximation seems to fit the data quite well. However, to be more exact, consideration may have to be taken of the relative sizes of pIY1 and pSF2124, which are 9.0 and 7.4Mdaltons respectively. It has been claimed that the plasmid copy-number, for a given ColE1 derivative or chimera, is inversely proportional to its size (*e.g.* see Gelfand *et al.*, 1978). This contention does not seem to have been proven and it may be that the type of regulation fixes the plasmid copy-number independent of its size.

auxotrophic strain of *E. coli* is to limit the concentration of a requisite amino acid.

IY35 has an absolute requirement for isoleucine plus valine.

As can be seen in Figure 3-1, a concentration of 0.1mM each of isoleucine plus valine limits the growth of IY35 to a Klett of 100. Consequently, IY35 was grown in 10L batch cultures under the same conditions as for IY13 (for details see Experimental section, Chapter 2) except that the final concentrations of isoleucine and valine were 0.1mM, and casamino acids were omitted. Growth ceased when a Klett of 100 was reached. The cultures were incubated under such conditions for 1, 2 and 4 hours before growth was restarted by the addition of 0.3mM each of isoleucine plus valine. Cells were harvested at Klett 200 (Figure 3-2).

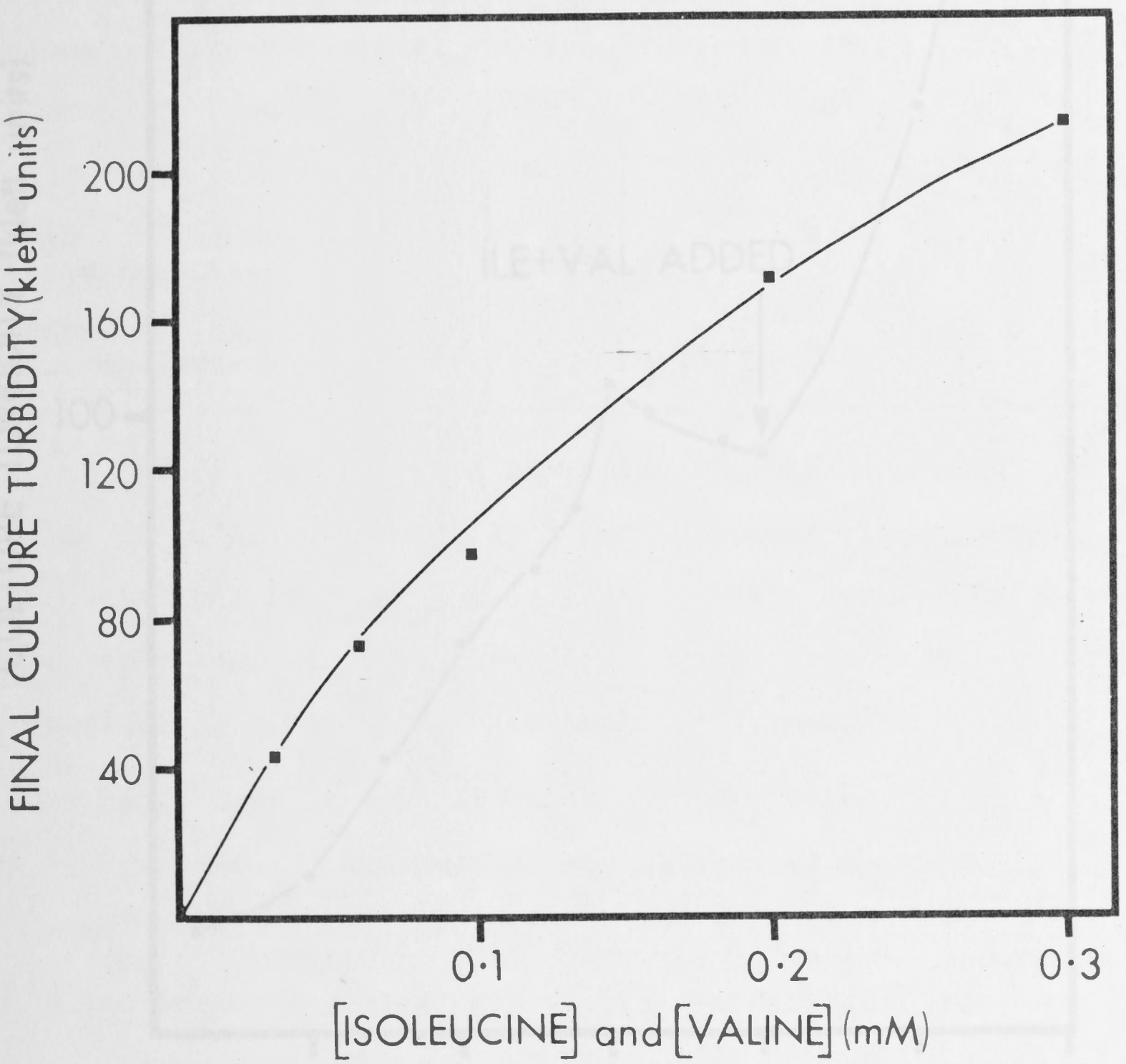
Membrane particles were then prepared from each batch of cells, and respiratory activities determined for both membrane and cytoplasmic fractions.

By using amino acid starvation to amplify the copy-number of the hybrid plasmid pIY1 in strain IY35, the levels of NADH:ubiquinone oxidoreductase in the membrane were found to be amplified up to 15-fold relative to the wild-type level, without appreciable amplification of the activity in the cytoplasm (Table 3-2).

Amplification of Enzyme Levels with Chloramphenicol

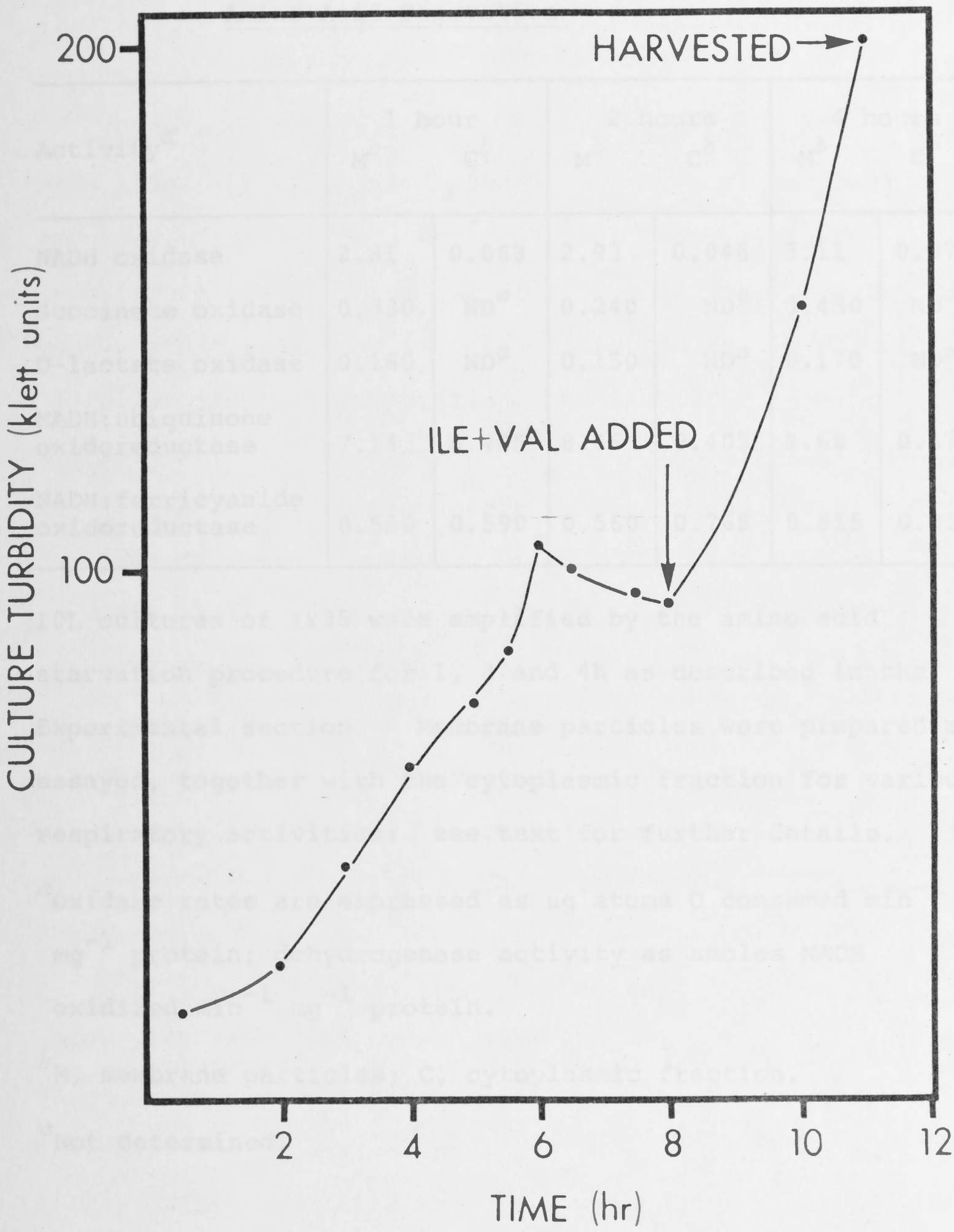
A potentially more effective way of increasing plasmid copy-number is to use the protein synthesis inhibitor, chloramphenicol. This antibiotic is routinely used to amplify plasmid levels when large amounts of DNA are required, *e.g.* to prepare plasmid DNA for cloning experiments.

FIGURE 3-1. Growth of IY35 Under Limiting Concentrations of Isoleucine and Valine.



10mL cultures of IY35 were grown in side-arm flasks at 37° in mannitol-minimal medium except that the final concentrations of isoleucine and valine were each at the indicated values. Growth ceased after 20h; the final culture turbidity reached is plotted against the concentration of the two limiting amino acids.

FIGURE 3-2. Amplification of NADH:ubiquinone Oxidoreductase Levels by Amino Acid Starvation.



10L cultures of IY35 were grown on mannitol-minimal medium except that the concentrations of isoleucine and valine were each 0.1mM. Growth ceased abruptly when a Klett value of 100 was reached: at this point, cultures were incubated for 1h, 2h (in the above instance) and 4h, and growth restarted by the addition of 3mmoles (per 10L culture) each of isoleucine and valine. Cells were harvested at a Klett value of 200 in each case and membrane particles prepared and assayed (see text).

TABLE 3-2 Respiratory Activities of Membrane and Cytoplasmic Fractions of IY35 Amplified by Amino Acid Starvation

Activity[a]	1 hour		2 hours		4 hours	
	M[b]	C[b]	M[b]	C[b]	M[b]	C[b]
NADH oxidase	2.81	0.063	2.93	0.046	3.11	0.076
Succinate oxidase	0.330	ND[c]	0.240	ND[c]	0.430	ND[c]
D-lactate oxidase	0.160	ND[c]	0.150	ND[c]	0.170	ND[c]
NADH:ubiquinone oxidoreductase	7.14	0.405	8.46	0.405	8.68	0.574
NADH:ferricyanide oxidoreductase	0.550	0.590	0.560	0.765	0.815	0.830

10L cultures of IY35 were amplified by the amino acid starvation procedure for 1, 2 and 4h as described in the Experimental section. Membrane particles were prepared and assayed, together with the cytoplasmic fraction for various respiratory activities: see text for further details.

[a]Oxidase rates are expressed as μg atoms O consumed min^{-1} mg^{-1} protein; dehydrogenase activity as μmoles NADH oxidized min^{-1} mg^{-1} protein.

[b]M, membrane particles; C, cytoplasmic fraction.

[c]Not determined.

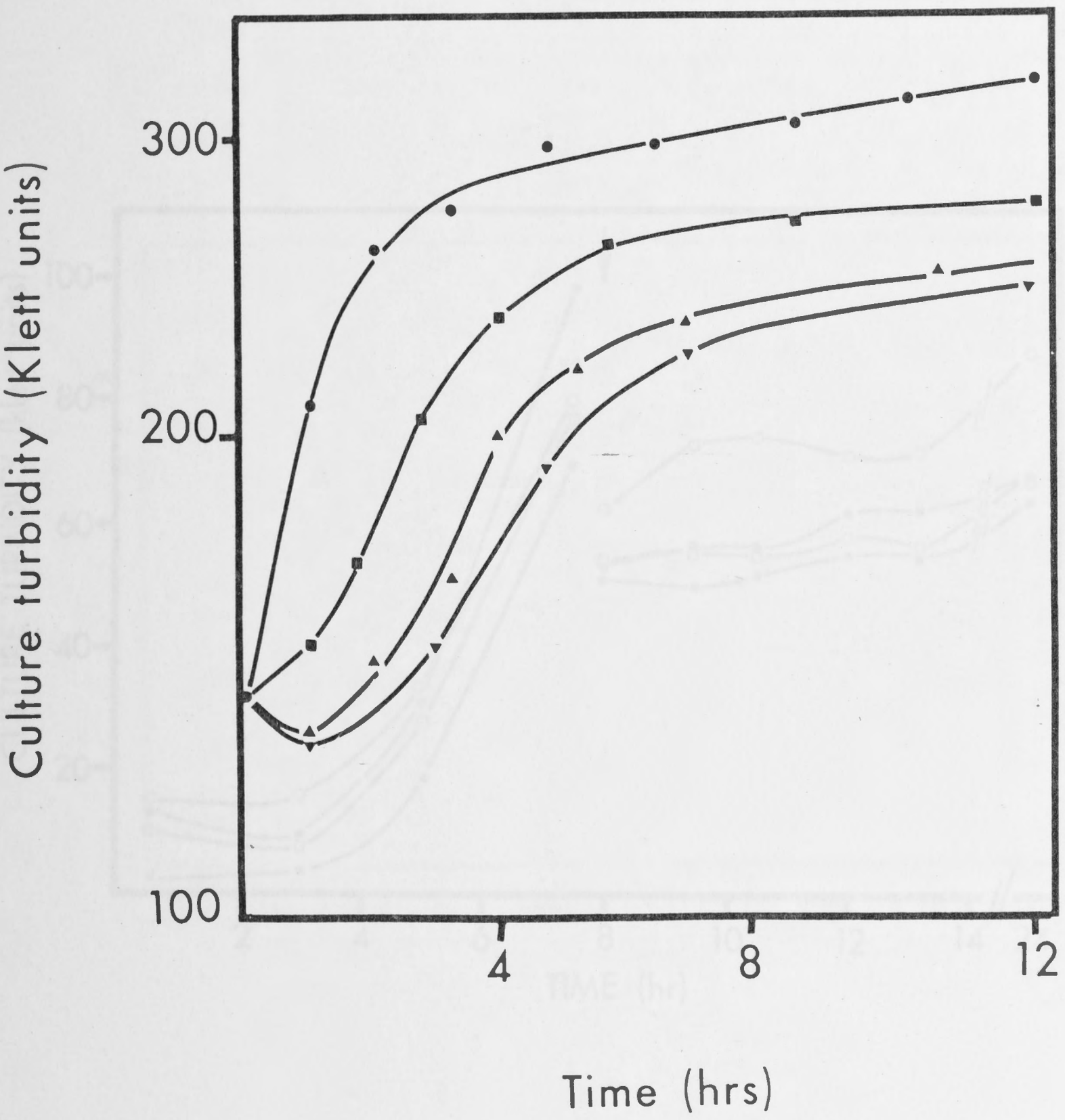
For it to be useful in amplifying enzyme levels however, conditions have to be found which allow subsequent recovery of protein synthesis after inhibition by chloramphenicol.

The experiments illustrated in Figure 3-3 show that even after 12h incubation with 150µg mL^{-1} chloramphenicol, growth of IY35 cultures could be restarted, without excessive lag, after removal of the inhibitor, suggesting that protein synthesis is being recovered in the majority of cells. It is also apparent that the longer the cultures are incubated in the presence of chloramphenicol, the longer is the lag period before growth restarts, and the longer the subsequent generation time.

To minimize the possible side-effects of incubation with chloramphenicol, the minimum concentration which produces bacteriostasis was determined (Figure 3-4). Slow growth was seen in the presence of 25µg mL^{-1} antibiotic, and from the results of Figure 3-4 a concentration of 50µg mL^{-1} was considered optimal and chosen for all subsequent experiments.

IY35 was grown in 10L batch cultures to Klett 100, then solid chloramphenicol added to a final concentration of 50µg mL^{-1}. Growth ceased almost immediately (Figure 3-5). Cultures were incubated for 4 and 9h with aeration and stirring then the cells harvested, washed in a small volume of sterile medium and used to reinoculate 10L of fresh medium. After one generation's growth the cells were harvested, then membrane particles prepared and assayed for various respiratory activities. This procedure results in an approximately 50-fold amplification of the levels of

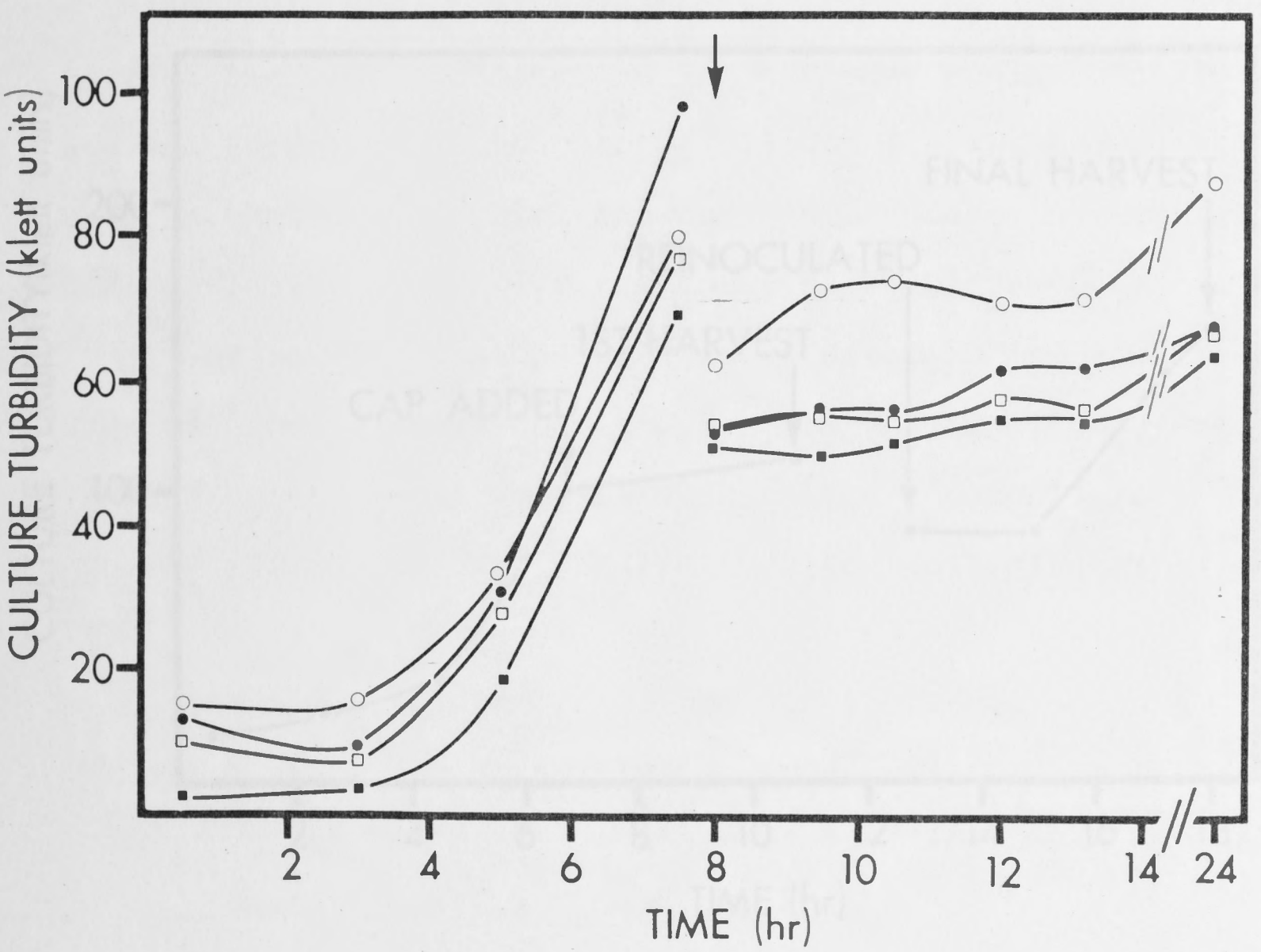
FIGURE 3-3. Effect of Incubation with Chloramphenicol on the Viability of IY35 Cultures



Cultures of IY35 were grown on mannitol-minimal medium to a Klett value of 135, then 150µg mL^{-1} chloramphenicol was added. At various time intervals 10mL aliquots were removed, the cells washed free of antibiotic and used to reinoculate fresh medium. Shown above is the recovery of growth after 0 ●——● 4 ■——■ 8 ▲——▲ and 12h ▼——▼ incubation with chloramphenicol.

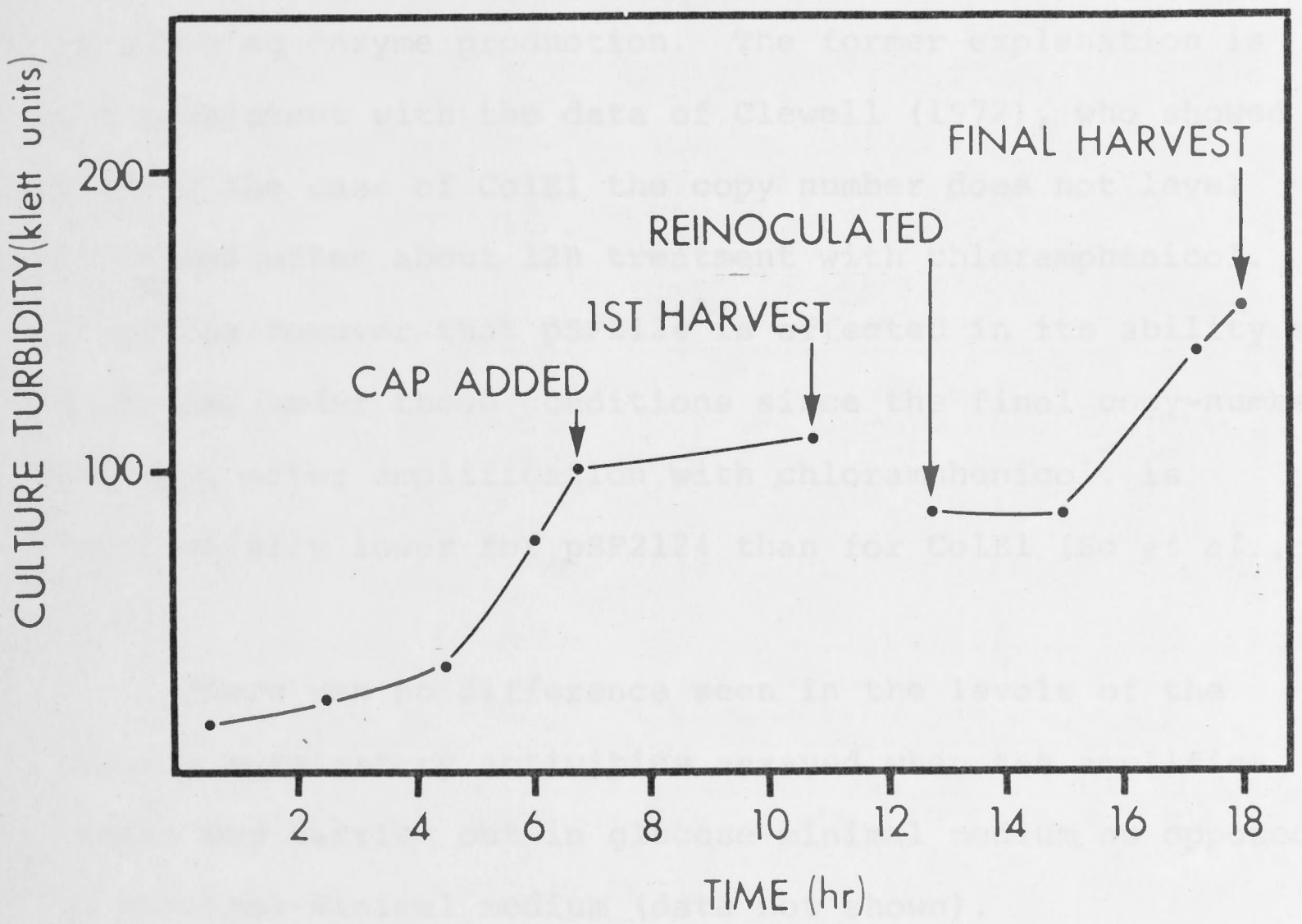
For details see Experimental section.

FIGURE 3-4. Optimum Inhibitory Concentration of Chloramphenicol.



4 x 10mL cultures of IY35 were grown on mannitol-minimal medium at 37°. When a Klett value of ~80 was reached, 10.0, 7.5, 5.0 and 2.5mL of sterile medium containing 200µg mL^{-1} chloramphenicol was added (at arrow) and the cultures adjusted to a final volume of 20mL with sterile medium. Subsequent growth was monitored for 15h: ○———○ 25µg mL^{-1}, □———□ 50µg mL^{-1}, ●———● 75µg mL^{-1}, ■———■ 100µg mL^{-1}.

FIGURE 3-5. Amplification of NADH:ubiquinone Oxido-reductase Levels by Chloramphenicol Treatment.



2x10L cultures of IY35 were grown on mannitol-minimal medium + 0.1% (w/v) casamino acids. When culture turbidity reached Klett 100, chloramphenicol was added to a final concentration of 50μg mL^{-1}, and the cultures incubated for 4h (as above) and 9h after which time the cells were harvested, washed in a small volume of sterile medium and used to reinoculate 10L of fresh medium. Growth restarted after a lag of 3 to 4h in both cases, and after allowing the culture to grow for about one generation the cells were harvested and membrane particles prepared and assayed.

NADH:ubiquinone oxidoreductase in the membrane (Table 3-3), without any appreciable increase in the specific activity in the cytoplasm.

There appeared to be no advantage in incubating for longer periods of time with chloramphenicol, under these conditions. Presumably this means that either the plasmid copy-number is already saturated after 4h treatment with chloramphenicol or that some factor other than gene-dosage is limiting enzyme production. The former explanation is not consistent with the data of Clewell (1972), who showed that in the case of ColE1 the copy number does not level off until after about 12h treatment with chloramphenicol. It may be however that pSF2124 is affected in its ability to replicate under these conditions since the final copy-number reached, after amplification with chloramphenicol, is substantially lower for pSF2124 than for ColE1 (So *et al.*, 1975).

There was no difference seen in the levels of the various respiratory activities assayed when the amplification was carried out in glucose-minimal medium as opposed to mannitol-minimal medium (data not shown).

In Table 3-4 the data are summarised from five separate chloramphenicol amplification experiments, and compared to those from three control membrane preparations. These results show that this procedure increases the NADH: ubiquinone oxidoreductase activity in the membrane over fifty-fold, and that furthermore there is a five-fold increase in the level of NADH oxidase. Data will be presented (see below) that this is not accounted for by an increase in the levels of the other measurable respiratory

TABLE 3-3 Respiratory Activities of Membrane and Cytoplasmic Fractions of IY35 Amplified with Chloramphenicol

Activity[a]	4 hour		9 hour	
	M[b]	C[b]	M[b]	C[b]
NADH oxidase	4.33	0.077	3.01	0.060
Succinate oxidase	0.399	ND[c]	0.317	ND[c]
D-lactate oxidase	0.112	ND[c]	0.064	ND[c]
NADH:ubiquinone oxidoreductase	27.9	0.635	28.4	1.000
NADH:ferricyanide oxidoreductase	1.87	0.550	2.87	0.615

10L cultures of IY35 were amplified by the chloramphenicol amplification procedure for 4 and 9h as described in the Experimental section. Membrane particles were prepared and assayed, together with the cytoplasmic fraction, for various respiratory activities: see text for further details.

[a] Oxidase activity expressed as μg atoms O consumed min^{-1} mg^{-1} protein, dehydrogenase activity as μmoles NADH oxidized min^{-1} mg^{-1} protein.

[b] M, membrane particles; C, cytoplasmic fraction.

[c] Not determined.

TABLE 3-4 Comparison of the Respiratory Rates of IY13 and Chloramphenicol-Amplified IY35 Membrane Particles

Activity	IY13 $\bar{x}$	IY13 s	IY35 $\bar{x}$	IY35 s
NADH oxidase	0.631	0.087	3.39	0.739
Succinate oxidase	0.280	0.116	0.304	0.105
D-lactate oxidase	0.253	0.031	0.130	0.067
NADH:ubiquinone oxidoreductase	0.508	0.119	26.1	3.371

Respiratory activities were determined on membrane particles prepared from 3 separate 10L cultures of IY13 and 5 separate chloramphenicol-amplified 10L cultures of IY35. The respective mean values and standard errors are tabulated. See text for further details.

components. This shows that the NADH:ubiquinone oxido-reductase is probably rate-limiting in the overall NADH oxidase pathway in wild-type cells, a situation opposite to that seen in the mitochondrion (Singer & Gutman, 1971), and that in chloramphenicol-amplified cells the activity of some other component becomes rate-limiting. This is supported by the fact that the levels of succinate and D-lactate oxidases are not enhanced.

Complementation of *Ndh* in IY36

The hybrid plasmid pIY2 carries a 4.6Mdalton DNA fragment inserted into the *Hin*dIII site of pGM706 (Young *et al.*, 1978). Strain IY36, which carries pIY2, is able to grow on medium with mannitol as sole C source (Table 3-6).

If pIY2 carries the gene coding for the respiratory NADH dehydrogenase, since it possesses the larger piece of DNA, either the 1.6Mdalton *Eco*Rl fragment in pIY1 is a subset of the 4.6Mdalton *Hin*dIII fragment, or they represent overlapping pieces of the chromosome. Restriction analysis of pIY1 and pIY2, however, has demonstrated that the two cloned DNA fragments do not have a region of common sequence (I.G. Young, unpublished results).

It was not clear how pIY2 complements *ndh* mutants, and, when the levels of various respiratory activities in membrane particles prepared from IY36 were examined, it was found that there is negligible NADH oxidase activity (Table 3-5), which agrees with the results of restriction analysis that the 4.6Mdalton *Hin*dIII fragment does not carry the

TABLE 3-5 Respiratory Activities in Membrane Particles Prepared from Strains IY36 and IY13

Activity[a]	IY13	IY36
NADH oxidase	0.78	<0.02
Succinate oxidase	0.09	0.06
D-lactate oxidase	0.10	1.03
D-lactate dehydrogenase	0.09	2.33

10L cultures of IY13 and IY36 were grown on mannitol minimal medium + 0.1% (w/v) casamino acids, and membrane particles were prepared and assayed for various respiratory activities as described in the text.

[a] Oxidase activities are expressed as µg atoms O consumed min^{-1} mg^{-1} protein. D-lactate dehydrogenase activity is expressed as µmoles MTT reduced min^{-1} mg^{-1} protein.

ndh gene*. The D-lactate oxidase activity is increased however 10-fold, and this is paralleled by a 25-fold increase in the D-lactate dehydrogenase activity. There is no change in succinate oxidase levels.

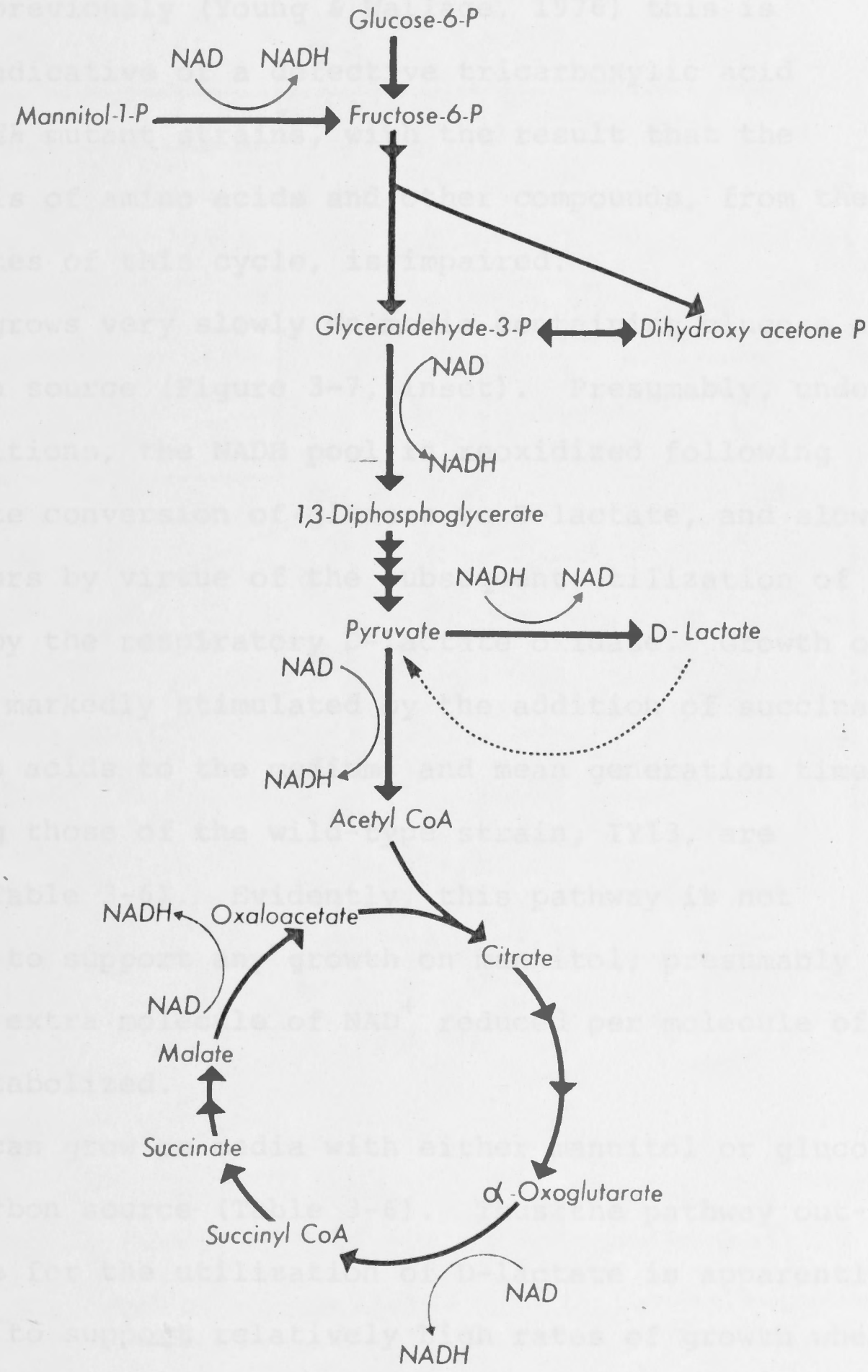
It seems likely that pIY2 contains the gene coding for the respiratory chain-linked D-lactate dehydrogenase flavoprotein. The amplification of this activity in IY36 membrane particles correlates with the augmentation of a protein band, seen in SDS polyacrylamide gel electrophoresis of IY36 membrane preparations, of apparent molecular weight 64,000 (Young, Jaworowski & Poulis, unpublished results).

To understand how the amplification of D-lactate dehydrogenase and D-lactate oxidase levels can overcome a mutational block in the membrane-bound NADH oxidase, let us consider the scheme shown in Figure 3-6. The soluble pyridine nucleotide-linked lactate dehydrogenase in *E. coli* is specific for the D(-) isomer of lactic acid (Tarmy & Kaplan, 1968). The equilibrium position for this reaction favours the formation of lactate. When coupled to the D-lactate oxidase reaction, this results in a 'futile cycle' whose net effect is that of NADH oxidation linked to the reduction of molecular O_2. Apparently the extent of this bypass pathway for NADH oxidation is not significant in wild-type cells (see below), but when the level of the D-lactate dehydrogenase flavoprotein is amplified by gene cloning, it becomes a major route for the regeneration of NAD^+.

---

*This statement presupposes the results of Chapter 4, where it is shown that the NADH:ubiquinone oxidoreductase amplifed in IY35 membranes is the respiratory NADH dehydrogenase.

FIGURE 3-6. A Bypass Route for NADH Oxidation in *E. coli*.



·····Amplified D-lactate oxidase (see text for details)

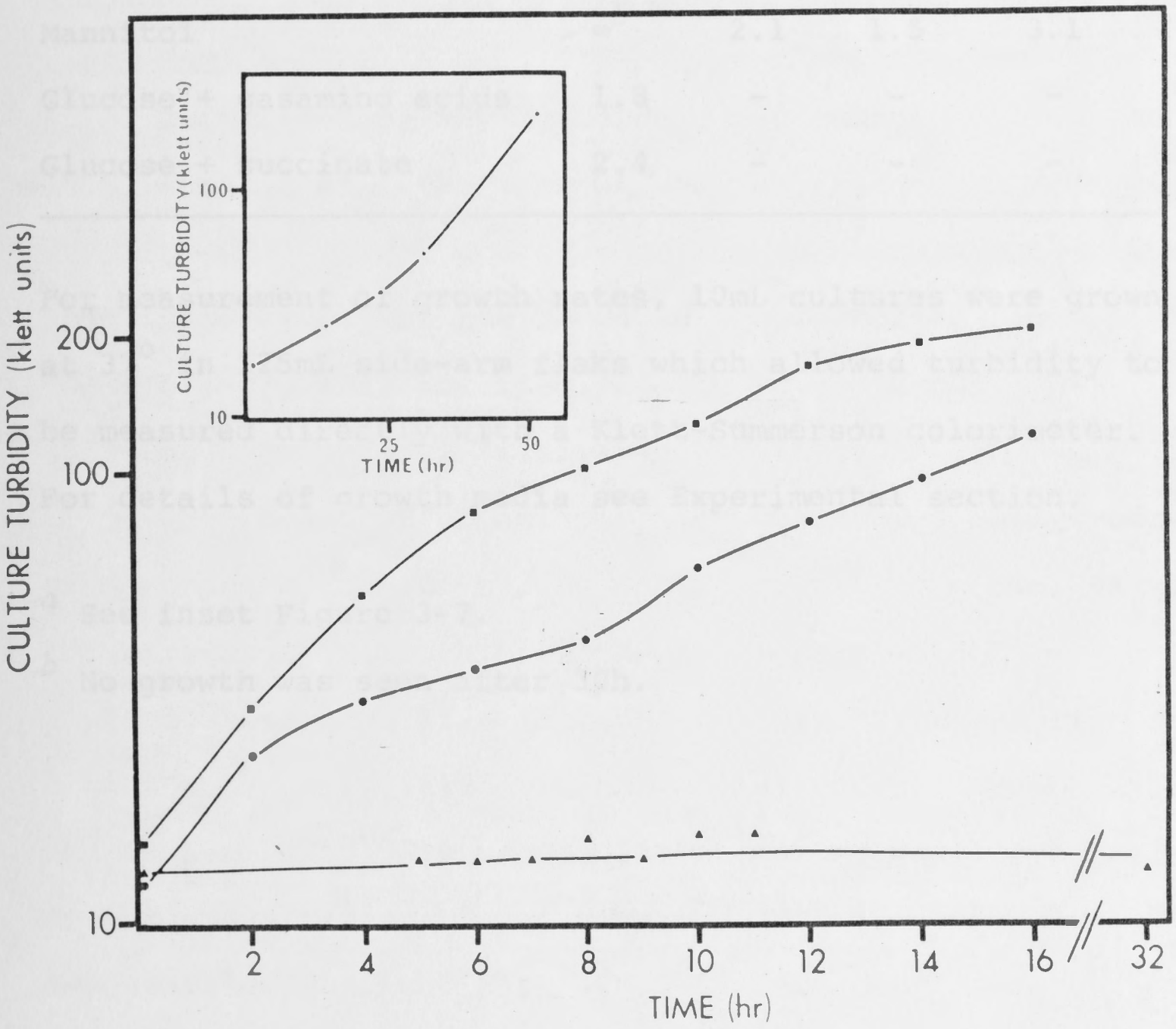
Growth Characteristics of IY12, IY13, IY35 and IY36

IY12 is unable to grow on media containing mannitol as sole carbon source unless the medium is supplemented with either succinate or casamino acids (Figure 3-7). As suggested previously (Young & Wallace, 1976) this is probably indicative of a defective tricarboxylic acid cycle in *ndh* mutant strains, with the result that the biosynthesis of amino acids and other compounds, from the intermediates of this cycle, is impaired.

IY12 grows very slowly on media containing glucose as sole carbon source (Figure 3-7, inset). Presumably, under these conditions, the NADH pool is reoxidized following the complete conversion of glucose to D-lactate, and slow growth occurs by virtue of the subsequent utilization of D-lactate by the respiratory D-lactate oxidase. Growth on glucose is markedly stimulated by the addition of succinate or casamino acids to the medium, and mean generation times approaching those of the wild-type strain, IY13, are obtained (Table 3-6). Evidently, this pathway is not sufficient to support any growth on mannitol; presumably due to the extra molecule of NAD^+ reduced per molecule of glucose catabolized.

IY36 can grow on media with either mannitol or glucose as sole carbon source (Table 3-6). Thus the pathway outlined above for the utilization of D-lactate is apparently sufficient to support relatively high rates of growth when the levels of the respiratory D-lactate dehydrogenase are amplified by gene cloning. The efficiency of this by-pass route for the reoxidation of NADH is less than that with the respiratory NADH oxidase itself however, since IY36

FIGURE 3-7. Growth of IY12 with Glucose or Mannitol as C Source.



For details see Experimental section. From the initial phase of growth a minimum doubling time of 2.1h was estimated for growth on mannitol + succinate and mannitol + casamino acids. Normal exponential growth was seen with glucose + succinate and glucose + casamino acids (for mean generation times see Table 3-6).
■———■ mannitol + casamino acids, ●———● mannitol + succinate, ▲———▲mannitol. Inset, glucose.

TABLE 3-6. Growth Rates of IY12, IY13, IY35 and IY36 on Mannitol and Glucose

Carbon Source	Mean Generation Time (h)			
	IY12	IY13	IY35	IY36
Glucose	slow[a]	1.9	1.5	2.8
Mannitol	∞[b]	2.1	1.5	3.1
Glucose + gasamino acids	1.8	-	-	-
Glucose + succinate	2.4	-	-	-

For measurement of growth rates, 10mL cultures were grown at 37° in 125mL side-arm flaks which allowed turbidity to be measured directly with a Klett-Summerson colorimeter. For details of growth media see Experimental section.

[a] See inset Figure 3-7.

[b] No growth was seen after 30h.

grows more slowly on mannitol or glucose compared to IY13 (Table 3-6).

As can be seen in Table 3-6, the high level of NADH: ubiquinone oxidoreductase and NADH oxidase does not impair the growth of IY35 on either mannitol or glucose.

Quinone and Cytochrome Content of *Ndh* Mutant, Wild-Type and Plasmid Strains

Estimation of the cytochrome and quinone content of IY12 revealed that the lack of the respiratory NADH oxidase in this strain is not due to a defect in the biosynthesis of ubiquinone or any of the detectable cytochromes, and is therefore probably due specifically to an inactive NADH dehydrogenase complex (Tables 3-7 & 3-8).

The levels of ubiquinone, menaquinone, demethylmenaquinone and cytochromes b_1, c and d are within normal values for strains IY35 (with or without chloramphenicol amplification) and IY36 (but see below). Thus the rise in the NADH and D-lactate oxidase levels seen in strains IY35 and IY36 respectively is not due to an increased synthesis of the other measurable respiratory components. This demonstrates that the NADH and D-lactate dehydrogenases are rate-limiting for their respective oxidases in wild-type cells. Upon amplification of either dehydrogenase in the membrane by the genetic techniques described above, an amplification of the relevant oxidase rate occurs until, presumably, the activity of some other component becomes rate-limiting. Alternatively a level may be reached where the excess dehydrogenase molecules cannot interact with the respiratory chain. In support of this, it has been found that the duroquinol oxidase activity in IY35 is not

TABLE 3-7. Cytochrome Content of Membrane Particles from Strains IY12, IY13, IY35 and IY36

Strain	Klett	Cytochrome[a]		
		b_1	o	d
IY12	200	0.203	0.088	0.085
IY13	40	0.222	0.081	0.035
"	200	0.159	0.087	0.053
IY35	200	0.163	0.057	0.030
cap.amp. IY35[b]	200	0.181	0.065	0.021
IY36	40	0.244	0.065	0.064
"	200	0.137	0.085	0.087

10L cultures were grown on mannitol-minimal medium + 0.1% (w/v) casamino acids to the indicated Klett value. Where indicated, cultures of IY35 were amplified with chloramphenicol for 4h as described in the text. For details of membrane preparation and cytochrome estimations see Experimental section.

[a] Cytochrome content expressed as nmoles mg^{-1} membrane protein.

[b] Amplified with chloramphenicol as described in Experimental section.

TABLE 3-8. Quinone Estimations on Whole Cells from Strains IY12, IY13, IY35 and IY36

Strain	Quinone[a]		
	UQ	MK	DMK
IY12	247	0.5	37.8
IY13	250	5.4	42.2
IY35	250	ND[b]	45.9
cap.amp. IY35[c]	218	12.5	28.9
IY36	180	26	42.9

10L cultures were grown on mannitol-minimal medium + 0.1% (w/v) casamino acids. For details of quinone extraction and estimation see Experimental section. Quinone content expressed as nmoles g^{-1} cells wet weight.

[a] UQ, ubiquinone; MK, menaquinone; DMK, demethylmenaquinone.

[b] ND, non detected.

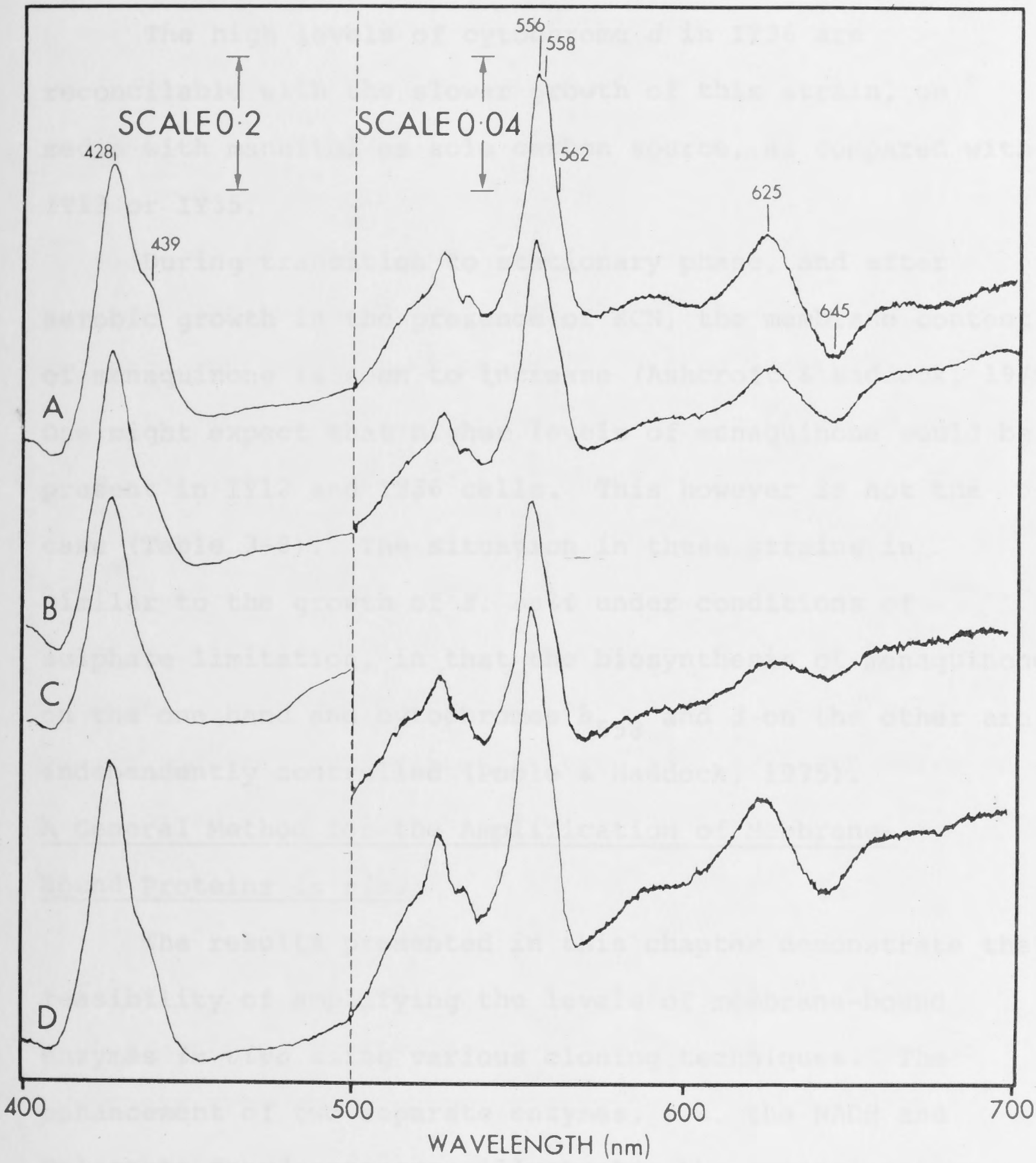
[c] Amplified with chloramphenicol as described in Experimental section.

amplified relative to that of IY13 (data not shown).

Closer examination of the results in Table 3-7 reveals an interesting observation: the cytochrome *d* levels in strains IY12 and IY36 are generally higher than those found in the control strain IY13, whereas the level in IY35, chloramphenicol amplified IY35, or cells grown to early exponential phase, is lower. High levels of cytochrome *d* are found in cells as cultures approach stationary phase (Castor & Chance, 1959; Shipp, 1972), and it has been speculated whether this may represent an adaptation by *E. coli* to conditions of low O_2 tension (Haddock & Jones, 1977). It has also been suggested that, under such conditions, *E. coli* synthesizes an alternative terminal respiratory complex consisting of cytochromes b_{558} and *d*, and that the synthesis of these two cytochromes is co-ordinately controlled (ibid.).

In the context of the above observation it is interesting to note that the response of cytochrome *d* synthesis to conditions of low oxygen tension is probably linked to the level of respiratory activity or some parameter associated with it, rather than O_2 tension *per se*, since a similar response is seen when cultures of *E. coli* are grown, aerobically, in the presence of sub-lethal concentrations of KCN (Ashcroft & Haddock, 1975) or under conditions of sulphate limitation (Poole & Haddock, 1975). Low temperature 'dithionite-reduced minus oxidized' difference spectra of membrane particles revealed discernable quantities of cytochrome b_{558} in IY12 (Figure 3-8(a)). This is not clearly seen in particles prepared from IY36, however (Figure 3-8(d)), suggesting that

FIGURE 3-8. Low Temperature 'Dithionite-Reduced Minus Oxidized' Difference Spectra of IY12, IY13, IY35 and IY36 Membrane Particles



'Dithionite-reduced minus oxidized' difference spectra of membrane particles were obtained at 77K as described in the Experimental section. Spectra were scanned between 400 and 700nm with the following instrument settings: full scale deflection, 0.5*A* (400 to 500nm) and 0.2*A* (500 to 700nm); spectral bandpass, 1nm; scan-rate, 1.0nm sec^{-1}. Indicated in spectrum (a) are the spectral positions of cytochrome *d* (peak 625nm, trough 645nm), cytochromes b_{556}, b_{558} and b_{562} and the Soret peaks of cytochromes *b* (428nm) and *a* (439nm). (a) IY12 membrane particles (14.7mg mL^{-1}). (b) IY13 membrane particles (17.3mg mL^{-1}). (c) IY35 membrane particles (15.0mg mL^{-1}). (d) IY36 membrane particles (13.4mg mL^{-1}).

synthesis of cytochromes b_{558} and *d* are not strictly linked under all conditions.

The high levels of cytochrome *d* in IY36 are reconcilable with the slower growth of this strain, on media with mannitol as sole carbon source, as compared with IY13 or IY35.

During transition to stationary phase, and after aerobic growth in the presence of KCN, the membrane content of menaquinone is seen to increase (Ashcroft & Haddock, 1975). One might expect that higher levels of menaquinone would be present in IY12 and IY36 cells. This however is not the case (Table 3-8). The situation in these strains is similar to the growth of *E. coli* under conditions of sulphate limitation, in that the biosynthesis of menaquinone on the one hand and cytochromes b_{558} and *d* on the other are independently controlled (Poole & Haddock, 1975).

A General Method for the Amplification of Membrane-Bound Proteins *in vivo*

The results presented in this chapter demonstrate the feasibility of amplifying the levels of membrane-bound enzymes *in vivo* using various cloning techniques. The enhancement of two separate enzymes, *i.e.* the NADH and D-lactate dehydrogenases, illustrates the general nature of the approach used. This is the first reported instance of the successful cloning of a gene coding for an intrinsic membrane-bound enzyme.

A technique has been developed to amplify the levels of NADH:ubiquinone oxidoreductase in the membrane over fifty-fold. No appreciable increase occurs in the activity seen in the cytoplasm, under the conditions used to prepare

the membrane particles, which suggests that the over-production does not lead to a saturation of binding sites for this enzyme in the membrane (however see below). The observed increase in NADH oxidase activity suggests that at least a proportion of the amplified enzyme interacts normally with the respiratory chain.

Since this work was published (Young *et al.*, 1978), a report has appeared detailing the overproduction of the membrane-bound enzyme, phosphatidylserine decarboxylase, in a strain of *E. coli* carrying a hybrid plasmid which has the *psd* gene inserted into ColE1 (Tyhach *et al.*, 1979). This strain was obtained by screening the Clarke and Carbon colony bank (Clarke & Carbon, 1976) for plasmids which complemented a *psd* point mutant following F-mediated conjugal transfer. Attempts were made to further increase the levels of phosphatidylserine decarboxylase by amplifying the plasmid copy-number with chloramphenicol. These attempts failed, presumably because there was no allowance made for the recovery of protein synthesis after the addition of the antibiotic. A similar degree of amplification, to the maximal levels reported above for NADH:ubiquinone oxido-reductase, was obtained by growing cultures of strains carrying the hybrid plasmid in medium containing a high concentration of valine. This has the effect of repressing the isoleucine and valine biosynthetic pathway, thereby starving the cells for isoleucine, and is a useful method of inducing amino acid starvation in strains which do not have a particular amino acid requirement. The amplified phosphatidylserine decarboxylase was reportedly inserted into the membrane at two distinct classes of binding site:

when membranes were prepared by osmotic lysis of spheroplasts, essentially all of the activity was membrane-bound, whereas when membrane particles were prepared by sonication, approximately 50% of the activity appeared in the cytoplasm. This was taken to mean that the normal membrane binding site is saturated by the overproduced enzyme, resulting in the binding of the remainder of the enzyme to a second, weaker site. If a similar situation exists upon amplification of NADH:ubiquinone oxidoreductase in IY35, it is not seen: it may be that disruption of cells in the French press does not dislodge enzyme from the membrane. No evidence to support the concept of specific binding sites for membrane-bound enzymes in general, was obtained by the amplification of either the NADH or D-lactate dehydrogenase.

It will be shown in the following chapter that the NADH:ubiquinone oxidoreductase amplified in IY35 cells is indeed the respiratory NADH dehydrogenase, *i.e.* the same enzyme whose activity is abolished in *ndh* mutant strains.

SUMMARY

During attempts to clone the gene coding for the respiratory NADH dehydrogenase, two hybrid plasmids were constructed, designated pIY1 and pIY2, both of which, when present in *ndh* mutant strains, confer the ability to grow on media with mannitol as sole carbon source (Young *et al.*, 1978).

Strains carrying pIY1 have 7 to 10-fold the wild-type level of NADH:ubiquinone oxidoreductase in their cell membranes, without any increase in the activity present in the cytoplasm.

A method has been developed which allows further amplification of the NADH:ubiquinone oxidoreductase level in the membrane to 50 times that found in wild-type cells. Under the conditions used, no amplification is seen in the activity present in the cytoplasmic fraction. NADH oxidase activity increases 4 to 6-fold without any increase in the levels of succinate or D-lactate oxidases.

Ndh mutant strains carrying pIY2 still lack membrane-bound NADH oxidase activity but have amplified D-lactate oxidase levels, presumably because pIY2 contains the gene coding for the respiratory D-lactate dehydrogenase flavoprotein. It is postulated that such strains have a novel pathway for NADH oxidation involving D-lactate oxidase and the soluble pyridine nucleotide-linked D-lactate dehydrogenase.

Examination of the quinone and cytochrome content of hybrid plasmid-containing strains suggests that the amplified levels of NADH and D-lactate oxidases in membrane particles prèpared from IY35 and IY36 respectively are probably due solely to the modulation of the activity of the relevant dehydrogenase in these strains.

CHAPTER 4.

Purification and Properties of the Respiratory NADH dehydrogenase from Genetically-Amplified Strains

INTRODUCTION

. In Chapter 2 it was shown that the *ndh* mutant strain, IY12, has less than 2% of the membrane-bound NADH oxidase activity compared to the control strain IY13, and that this can be correlated with the disappearance of a peak of NADH: ubiquinone oxidoreductase activity seen in chromatograms of cholate-solubilized IY13 membranes separated by hydroxyl-apatite column chromatography.

Following the construction of hybrid plasmids which complement *ndh* point mutants, a method has been described in Chapter 3 to amplify the levels of NADH:ubiquinone oxido-reductase up to 50-fold in the membrane of a strain (IY35) carrying one such plasmid, pIY1. In this chapter it is shown that the enzyme whose levels have been amplified in IY35 is in fact the respiratory NADH dehydrogenase. The purification and characterization of this enzyme complex is presented.

Most of the work has been carried out with enzyme preparations purified from strain IY35, and using the chloramphenicol amplification procedure, described in Chapter 3, to prepare membrane particles with a very high specific activity. During the course of this work another approach was developed to achieve even higher levels of NADH:ubiquinone oxidoreductase in the membrane. This involved the insertion of a piece of DNA containing two copies of the highly efficient *lac* promoter region into pIY1, presumably at the 5' end of the *ndh* gene, in an attempt to amplify enzyme levels by a combination of the

effects of both increased gene copy-number and an increased efficiency of transcription. To do this, plasmids pIY1 and pLJ3 (which was the source of the double *lac* promoter, Johnsrud (1978)) were separately digested with the restriction endonuclease *Eco*R1, and then ligated together (M.I. Poulis & I.G. Young, unpublished results). The ligation-mix was then transformed into IY12, and selection made for complementation of the *ndh* mutant phenotype on mannitol-minimal plates. The viable colonies were replated onto selective plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside ('x-gal'). Strains carrying hybrid plasmids which contain the double *lac* promoter fragment produce blue colonies since they are constitutive for the *lac* operon and therefore hydrolyze the non-inducing, chromogenic substrate (Johnsrud, 1978). Such an isolate, found to possess the highest NADH:ubiquinone oxidoreductase levels, was selected for further study, and designated IY85 and the plasmid it contains, pIY9.

Ongoing studies in the laboratory required large amounts of pure enzyme; consequently, in collaboration with Dr H.D. Campbell, the use of strain IY85 for the large-scale purification of NADH:ubiquinone oxidoreductase, was investigated.

EXPERIMENTAL

Media and Growth of Bacteria

For details of the medium used and the growth of 10L cultures in glass fermenters, see Experimental section, Chapter 2. 10L cultures of IY35 and IY85 were amplified with chloramphenicol as described in Chapter 3.

Purification of NADH:ubiquinone Oxidoreductase

For the preparation of highly purified NADH:ubiquinone oxidoreductase from chloramphenicol amplified IY35 or IY85, identical procedures to those used for the preparation of partially purified enzyme from IY13 were used (see Experimental section, Chapter 2). Peak fractions of NADH: ubiquinone oxidoreductase were pooled, frozen in liquid N_2 and stored at -15^{o}.

Large Scale Growth of Bacteria

A 40L batch culture of strain IY85 was grown in the stainless steel vessel of a New Brunswick Fermacell fermenter (model CF-50), with aeration (30L min^{-1}) and stirring (200 rpm). When the culture reached a Klett of 200, 2g of chloramphenicol was added and the culture incubated, typically for 8h, under the same conditions as above. The cells were then harvested, resuspended in a small volume of sterile medium, and used to inoculate 9 x 10L lots of fresh medium in 14L New Brunswick glass fermenters (to a starting Klett of approximately 100). The cultures were grown for one generation, exactly as for the small-scale work and the cells harvested (yield approximately 150g wet weight). The Fermacell was also used in the continuous culture mode to grow 240L quantities of IY85 at Klett 200.

Large-Scale Enzyme Purification

The preparation of membrane particles from the large-scale chloramphenicol-amplified IY85 cultures was essentially a scaled-up version of the small-scale procedure. Washed, resuspended membrane particles from one large-scale growth of cells were placed in a beaker, on ice, and 20%

(w/v) cholate and solid KCl were slowly added to the stirred solution to give final concentrations of 3% (w/v) cholate and 1M KCl. The mixture was centrifuged for 2h at 60,000 rpm in a Beckman 60Ti rotor. The resulting supernatant was loaded onto a column of hydroxylapatite (5 x 25-30cm) equilibrated with 4L of 20mM potassium phosphate buffer, pH 7.5, containing 0.1% (w/v) cholate and 20μM FAD. A 4L linear gradient; 0.02-1M potassium phosphate buffer, pH7.5, containing 0.1% (w/v) cholate and 20μM FAD, was applied immediately at a flow-rate of 150-200mL h^{-1} and 25mL fractions were collected. The pooled enzyme fractions were frozen in liquid N_2 in 50mL screwcap polypropylene centrifuge tubes and stored at -60^{o}.

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis on 10% and 15% polyacrylamide slab gels was carried out as described in Chapter 2. Gels were also used in which the separation gel contained a linear 10-25% concentration gradient of acrylamide.

For the determination of apparent subunit molecular weight, the following protein standards were used: γ-globulin (human), catalase (bovine liver), ovalbumin (chicken), L-lactate dehydrogenase (rabbit muscle), hexokinase (yeast), pyruvate kinase (rabbit muscle), serum albumin (bovine), phosphoglyceromutase (rabbit muscle), myoglobin (horse muscle), lysozyme (chicken), ferritin (horse spleen) and cytochrome c (horse heart). All were of the highest grades commercially available. Subunit molecular weight values were obtained from Weber and Osborn (1969) and Darnall and Klotz (1975). The logarithm of the subunit

molecular weight was plotted against R_f^{-1}, and estimates of the subunit molecular weight of NADH:ubiquinone oxidoreductase were obtained from the standard curve of the above data fitted by the least squares method.

Determination of FAD

A 20mL solution of pure enzyme (0.316mg mL^{-1}, specific activity 532 units mg^{-1}) was dialyzed against 90 volumes (nominal) of 5mM potassium phosphate buffer, pH 7.5, 0.1% (w/v) cholate (twice recrystallized, as described in Chapter 2), for 24h with one change of dialysis buffer. The volume of the solution after dialysis was determined gravimetrically, and the protein concentration by the procedure of Lowry *et al.* (1951) (see Chapter 2) and corrected according to the factor determined in Chapter 5 to give a true protein concentration of 0.168mg mL^{-1}. The molarity of the solution, with respect to the enzyme subunit, was determined from the molecular weight predicted from the protein sequence (see Chapter 5).

Total flavin was estimated fluorometrically according to the procedure of Bessey *et al.* (1949) with FAD (Sigma Chemical Co., St. Louis, Mo) as standard. The FAD stock solution (72.5μM) was standardized spectrophotometrically at 450nm using ε = 11,300cm^{-1} M^{-1}. Fluorescence measurements were made with a Perkin Elmer spectrofluorometer (model 6000) with the excitation wavelength set, nominally, at 450nm and the emission wavelength set, nominally, at 535nm. Measurement of the concentration of flavin in the dialyzed enzyme sample was made in triplicate using 0.3mL aliquots and corrected for the determined flavin concentration of the final dialyzate (approximately 2% of

the sample value).

Visible and ultraviolet spectra of the dialyzed enzyme preparation were obtained using a Cary 118C spectrophotometer, in 3mL matched quartz cuvettes. The reference cuvette contained a sample of the final dialyzate as blank, and the baseline, obtained with aliquots of dialyzate in sample and reference cuvettes, was manually subtracted to give the resulting spectra, depicted in Figure 4-8(a). The appropriate extinction coefficients at each wavelength were calculated from the molarity of the protein solution determined as described above.

A 2mL aliquot of dialyzed enzyme, sampled with an 'A-grade' bulb pipette, was extracted with 3 x 2mL of chloroform/methanol, 2:1 (v/v). The lipid extract was rotary evaporated then resuspended in 2mL of absolute ethanol. The final volume (1.98mL) was determined gravimetrically assuming a density of 0.79g cm^{-3}. The ultraviolet spectrum of the extract was obtained, using a Cary 118C spectrophotometer, against absolute ethanol. Apparent extinction coefficients for the lipid extract were calculated relative to the molarity of the protein making a correction for the minor volume change.

Extraction and Estimation of Lipid

For each determination, the pooled hydroxylapatite column fractions from one small-scale preparation were dialyzed for at least 24h at 4^{o} against 3 changes of glass-distilled water (40 volumes), then freeze dried. The freeze dried material was resuspended in 5mL of distilled water and extracted 4 to 6 times with an equal volume of chloroform/methanol, 2:1 (v/v). The lipid extract was then extracted

once with 2mL distilled water and analyzed.

Phospholipid P was estimated by measuring enhancement of the natural fluorescence of rhodamine 6G according to the method of Schiefer & Neuhoff (1971) and using *E. coli* phosphatidylethanolamine (Sigma Chemical Co., St. Louis, Mo.) as standard. The lipid extract was also analyzed for total phosphorus and estimated by dry weight determination after evaporation of the solvent and drying *in vacuo* to constant weight. The latter two analyses were kindly performed by Dr J. Fildes of the A.N.U. Analytical Services Department.

Chromatography of extracted lipids was carried out on silica gel thin layer plates (Merck, F_{254}, 0.5mm thick) using chloroform/methanol/H_2O, 65:31:4 (v/v) and chloroform/methanol/NH_3, 65:30:5 (v/v) as solvents. Lipids, in general, were visualized with iodine vapour, and lipids containing a free amino group (such as phosphatidylethanolamine) by spraying plates with ninhydrin (0.2% (w/v) in acetone).

Reconstitution of *Ndh* Mutant Membrane Particles

Two methods were used to reconstitute NADH oxidase from *ndh* mutant membrane particles and purified NADH:ubiquinone oxidoreductase.

In the first method, 0.05mL aliquots of membrane particles were placed in glass vials at 0-4°, and varying amounts of pure enzyme (0 to 100µL of pooled hydroxylapatite column fractions, ~30 units mL^{-1}) were added, and mixed thoroughly. 5µL of the mixture was assayed for NADH oxidase in an assay mixture containing 1mL 50mM TES buffer, pH 7.5, 40µM FAD and 250µM NADH, preincubated at 30°. The reaction was started by the addition of reconstituted

membrane particles and followed at 340nm. The rate of NADH oxidation was calculated assuming ε = 6,200cm^{-1} M^{-1}.

In the second method, 1mL of assay buffer (50mM TES buffer, pH7.5, 40μM FAD), 250μM NADH and 5μL of *ndh* mutant membrane particles were placed in a 1mL quartz cuvette and preincubated at 30°. Various volumes of pure enzyme (0 to 100μL of pooled hydroxylapatite column fractions, ~30 units mL^{-1}) were added, and NADH oxidation followed as above.

The rates of NADH oxidation obtained in both cases were corrected for the NADH oxidase activities of the mutant membrane particles and the pure enzyme, measured individually in the above assay system.

The cyanide sensitivity of the various NADH oxidase activities was measured after the addition of 3μL of a 1M KCN solution.

Method 2 was used routinely to test reconstitution of *ndh* mutant particles under different conditions. To determine the location of the reconstituted NADH oxidase, 0.5mL of IY1-2 membrane particles (44mg mL^{-1}) was reconstituted, using method 1, with 250μL of pure enzyme (28.5 units mL^{-1}), then diluted with 3.45mL of STM buffer, and centrifuged for 3h at 48,000 rpm in a Spinco SW56 rotor. The pellet was resuspended in 5mL of the same buffer then assayed, together with the soluble fraction, for NADH oxidase, as above, and NADH:ubiquinone oxidoreductase.

Metal Analyses and Other Assays

Analysis of the metal content of NADH:ubiquinone oxidoreductase was done, following the development of large-scale preparation of enzyme, in conjunction with Dr H.D. Campbell who performed the analyses.

The Mo and W content of the pure enzyme preparation was measured after digesting the pooled hydroxylapatite column fractions with H_2SO_4 and H_2O_2 as described by Cardenas and Mortenson (1974). Digestions were performed in 3mL stoppered quickfit tubes as suggested by Beinert (1978). Cu was assayed, after wet ashing of pooled column fractions, according to Beinert (1978). Total Fe and non-haem iron were assayed essentially by the method of Doeg and Ziegler (1962) on 400µL and 100µL aliquots respectively of individual hydroxylapatite column fractions, and acid-labile sulphur assayed on the same material (Rabinowitz, 1978).

Procedures for the determination of protein by the method of Lowry *et al.* (1951), and for enzyme assays, have been described in Chapter 2.

RESULTS AND DISCUSSION

Purification of NADH:ubiquinone Oxidoreductase from Chloramphenicol-Amplified IY35 Cultures

Membrane particles were prepared, washed and solubilized as described in Chapter 2 from chloramphenicol-amplified IY35 cultures. The apparent recovery of NADH: ubiquinone oxidoreductase activity during solubilization was generally higher than obtained, under identical conditions, with IY13 membrane particles (Table 4-1). This appears to be due to a partial inhibition of activity, by potassium cholate, which is less apparent with the procedures used in assaying IY35 solubilized preparations: even though identical conditions are used to assay both wild-type and the highly amplified preparations, the very

TABLE 4-1. Purification of NADH:ubiquinone Oxidoreductase from Chloramphenicol-amplified IY35 Membrane Particles[a]

	Volume mL	Activity[b] (Units mL^{-1})	(Units)	Protein (mg mL^{-1})	(mg)	Specific Activity (Units mg^{-1})	Yield (%)	Purification (fold)
Membrane Particles	12.5	1027	12844	36.6	457	28.1	(100)	(1)
Cholate-soluble	10.5	1152	12093	12.1	127	95.2	94	3.4
Hydroxylapatite	75.0	41.1	3083	0.077	5.8	531	24	19

[a] IY35 cultures were amplified with chloramphenicol as described in Chapter 3. Details of purification are given in Chapter 2 and are identical to those used for the preparation of partially purified enzyme from IY13 membrane particles.

[b] 1 unit ≡ 1 μmole NADH oxidized min^{-1}. For details of assay see text.

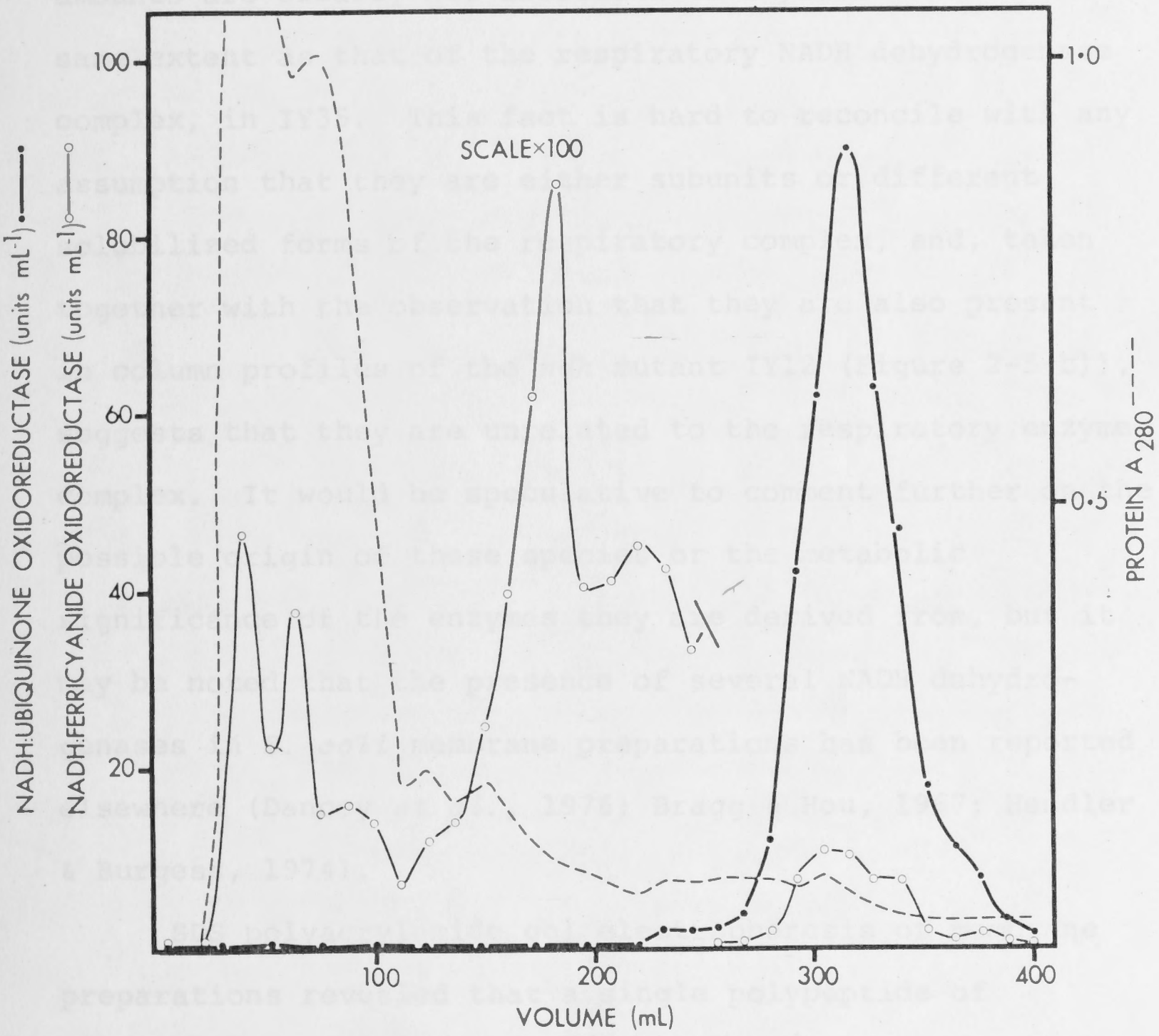
high levels of enzyme in the latter necessitate diluting the sample assayed at least 100-fold for accurate determination of activity, and this probably has the effect of relieving the slowly-reversible inhibition by cholate (Figure 2-3).

The cholate-soluble material was chromatographed on hydroxylapatite under conditions identical to those described in Chapter 2, and column fractions were assayed for protein (A_{280}), NADH:ferricyanide oxidoreductase and NADH:ubiquinone oxidoreductase (Figure 4-1). The chromatograms reveal a very large increase in the size of the NADH:ubiquinone oxidoreductase peak. Remembering that equivalent amounts of membrane particles were solubilized in all cases, this indicates that the enzyme whose levels are amplified in IY35 membranes, and the gene for which has been cloned into pIY1, is the respiratory NADH dehydrogenase*, identified as the enzyme inactivated by the mutation in IY12. Furthermore, the overproduced enzyme behaves chromatographically in an identical manner to the enzyme obtained from wild-type cells. No detectable activity with NADPH as electron donor was seen across the amplified peak; an upper limit can be set such that under identical assay conditions the activity with NADPH as electron donor is less than 0.02% that with NADH as electron donor.

---

* This statement anticipates later results which show that the enzyme complex actually consists of a single type of polypeptide chain (see below and also Chapter 5).

FIGURE 4-1. Hydroxylapatite Column Chromatography of Cholate-Solubilized Membrane Particles from Chloramphenicol-Amplified IY35.



For details see Experimental section. Further details are given in Figure 2-5.

In other respects, the chromatograms depicted in Figures 2-5(a) and 4-1 are similar, in particular with regards to the amount of the other NADH:ferricyanide oxidoreductases resolved during chromatography. Although there were variations seen in the sizes of the peaks corresponding to these enzymes between different experiments, their amounts are clearly not increased to anywhere near the same extent as that of the respiratory NADH dehydrogenase complex, in IY35. This fact is hard to reconcile with any assumption that they are either subunits or different solubilized forms of the respiratory complex, and, taken together with the observation that they are also present in column profiles of the *ndh* mutant IY12 (Figure 2-5(b)), suggests that they are unrelated to the respiratory enzyme complex. It would be speculative to comment further on the possible origin of these species or the metabolic significance of the enzymes they are derived from, but it may be noted that the presence of several NADH dehydrogenases in *E. coli* membrane preparations has been reported elsewhere (Dancey *et al.*, 1976; Bragg & Hou, 1967; Hendler & Burgess, 1974).

SDS polyacrylamide gel electrophoresis of membrane preparations revealed that a single polypeptide of apparent molecular weight 45,000 (see below) is present in significantly increased amounts in chloramphenicol-amplified IY35 membranes as compared to membranes from IY13 (Figure 4-2). When the peak fractions, after hydroxylapatite chromatography, of the respiratory NADH:ubiquinone oxidoreductase, were pooled and electrophoresed, it was found that this enzyme consists of a single polypeptide

FIGURE 4-2. SDS-polyacrylamide Gel Electrophoresis of Membranes Prepared from Wild-Type and Plasmid-Containing Cells and Pure NADH: ubiquinone Oxidoreductase.

Samples: (a) and (e), pure NADH:ubiquinone oxidoreductase (see text); (b), IY13 membrane particles; (c), IY34 membrane particles; (d), chloramphenicol-amplified IY35 membrane particles.

species with an apparent molecular weight coinciding with that of the polypeptide whose level is increased in IY35 membranes. It appears, therefore, that the respiratory NADH dehydrogenase complex is an enzyme consisting of a single polypeptide type*, in marked contrast to the corresponding enzyme complex from mitochondria.

As can be seen in Table 4-1, the NADH:ubiquinone oxidoreductase has been purified 19-fold over the activity found in chloramphenicol-amplified IY35 membrane particles, which corresponds to a purification of 800 to 1000-fold relative to wild-type membrane particles.

The mitochondrial NADH:ubiquinone oxidoreductase has been isolated as a large lipoprotein complex (complex 1) with an estimated molecular weight of approximately 850,000 (Hatefi & Stigall, 1976). Reports vary on the actual polypeptide composition of this complex, but it has been reported to contain at least 10 (Capaldi, 1974; Hatefi & Stempel, 1969) and as many as 16 (Ragan, 1976(b)) or even 26 (Heron *et al.*, 1979) *different* polypeptides.

Taken at face value, the evidence points to the *E. coli* NADH:ubiquinone oxidoreductase as being much simpler in construction compared to the corresponding mitochondrial enzyme. There are three possible explanations for this apparent difference:

(1) The two enzymes are fundamentally different in structure, which underlies a basic difference in their metabolic roles or mechanism of catalysis.

---

*Evidence will be presented in Chapter 5 that the enzyme does not consist of two dissimilar polypeptides of the same molecular weight.

(2) The enzyme purified from IY35 is the primary NADH dehydrogenase of *E. coli*, but can reduce ubiquinone *in vitro;* however, electron transport between NADH and ubiquinone in the membrane involves other, additional polypeptides.

(3) The structure of the mitochondrial NADH:ubiquinone oxidoreductase is very much simpler than that of complex 1, and many of the polypeptides present in complex 1 are not involved in electron transport between NADH and ubiquinone.

Alternatives (2) and (3) imply that the differences between the mitochondrial and *E. coli* NADH:ubiquinone oxidoreductases are only apparent. The second alternative is not considered a valid proposition, and this point will be taken up again in Chapter 6.

The specific activity of the *E. coli* NADH:ubiquinone oxidoreductase, approximately 530μmoles NADH oxidized min^{-1} mg^{-1}, at 30^{o} (Table 4-1), is the highest reported so far with ubiquinone as electron acceptor, and it is therefore unlikely that the activity measured is artifactual. The turnover number assuming one active site per polypeptide chain, under the assay conditions used, 2.51×10^{4} min^{-1} (30^{o}, pH7.5) is similar to that of complex 1, admittedly under different assay conditions, of 2.12×10^{4} min^{-1} (38^{o}, pH8.0) assuming one active site for complex 1 for a molecular weight of 850,000. The specific activity of complex 1 used for the calculation was taken as 25μmoles NADH oxidized min^{-1} mg^{-1} (Hatefi & Stempel, 1969).

Complex 1 is possibly the only solubilized preparation from the mitochondrion, to-date, which catalyzes a

physiologically meaningful NADH:ubiquinone oxidoreductase activity (see Chapter 1), but it is still not clear, despite intense investigation in many laboratories, how many polypeptides are involved in the electron transfer between NADH and ubiquinone on the mitochondrion. Without this knowledge, a meaningful comparison between the enzymes from *E. coli* and mitochondria is not possible.

The specific activity of the pure NADH:ubiquinone oxidoreductase from chloramphenicol-amplified IY35 is in close agreement with that estimated for the enzyme in the partially purified preparation from IY13, which is based on the assumption that there is one active site per polypeptide of apparent molecular weight 45,000 (Chapter 2). This supports the assumption that the catalytic centre activity of the enzyme has not been altered by the techniques employed to amplify its levels in the membrane. Taken in conjunction with the evidence from SDS polyacrylamide gel electrophoresis (Figure 4-2) this shows that the increase in the specific activity of IY35 or chloramphenicol-amplified IY35 membranes, compared to IY13 membranes, is due to a parallel increase in the number of enzyme molecules, and not due to activation. The argument can be advanced that the NADH:ubiquinone oxidoreductase is a multisubunit enzyme, but that only the content of the primary dehydrogenase has been increased and that this has been subsequently purified (*e.g.* see case 2 above). It is unlikely that such an enzyme preparation would have the same specific activity as the stoichiometric complex, and this is not considered a tenable proposition.

The cloned DNA insert is of sufficient size (1.6M daltons or approximately 2,600 base pairs) to code for a maximum of approximately 800 amino acids, or the equivalent of a protein with a molecular weight about 90,000. It does not seem likely, therefore, that the DNA fragment contains the structural genes coding for two separate polypeptides of identical molecular weight, and indeed that this is not so will be shown in Chapter 5.

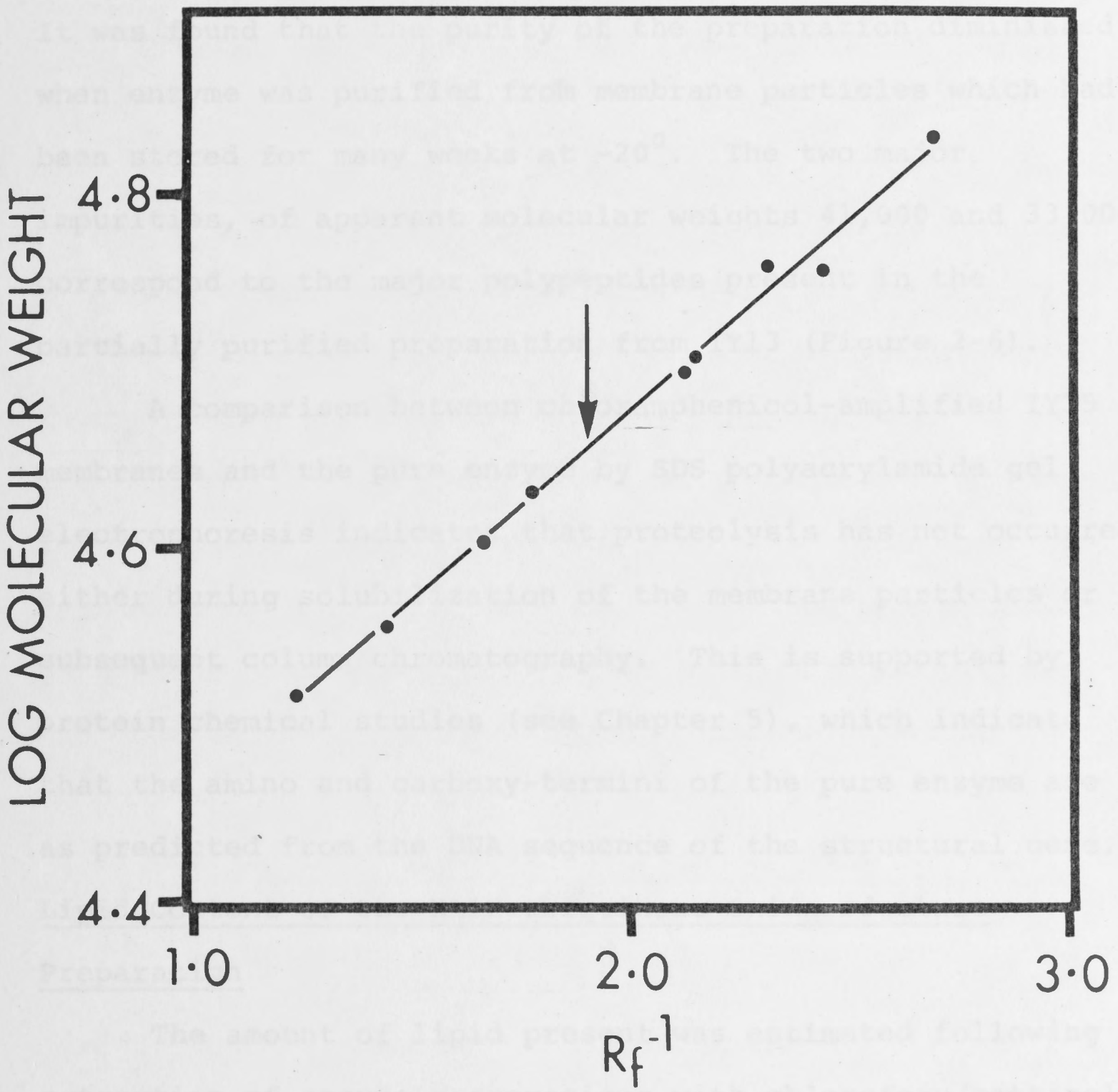
Estimation of the Subunit Molecular Weight of NADH:ubiquinone Oxidoreductase

The subunit molecular weight of the pure enzyme was estimated by SDS polyacrylamide gel electrophoresis using the same gel system employed for analytical separation. It was found that plots of the logarithm of apparent molecular weight versus R_f, for various standard proteins, are not linear with this system, using either 10% or 15% acrylamide gels. Plots of the logarithm of apparent molecular weight versus R_f^{-1} are linear, however, within the molecular weight range 30,000 to >70,000, and such plots, fitted by the least squares method, were routinely employed for molecular weight estimation (Figure 4-3). The subunit molecular weight, calculated from the results of four separate experiments, was estimated to be 44,600 (S.E. = 1,200).

Experiments with 10% and 15% polyacrylamide gels showed that the apparent molecular weight estimated by this technique is not significantly altered by increasing the degree of cross-linking in the gel, as is the case for some membrane-bound proteins (Tanner, 1979).

There are traces of impurities of lower molecular

FIGURE 4-3. Molecular Weight Estimations by SDS-polyacrylamide Gel Electrophoresis.



Details are given in the Experimental section. Linear estimation of the subunit molecular weight of NADH: ubiquinone oxidoreductase on such plots in four separate experiments (3 on 10% and 1 on 15% polyacrylamide gels) gave values of 45,600, 45,500, 43,000 and 44,400 (mean = 44,600, S.E. = 1200).

weight, as well as material migrating at the dye front, visible on 10% acrylamide gels (Figure 4-2). The latter material is not present on 15% acrylamide gels, and may be an artifact: the major band accounts for 90 to 95% of the material included in 10% or 15% polyacrylamide gels. It was found that the purity of the preparation diminished when enzyme was purified from membrane particles which had been stored for many weeks at -20°. The two major impurities, of apparent molecular weights 41,000 and 33,000, correspond to the major polypeptides present in the partially purified preparation from IY13 (Figure 2-6).

A comparison between chloramphenicol-amplified IY35 membranes and the pure enzyme by SDS polyacrylamide gel electrophoresis indicates that proteolysis has not occurred either during solubilization of the membrane particles or subsequent column chromatography. This is supported by protein chemical studies (see Chapter 5), which indicate that the amino and carboxy-termini of the pure enzyme are as predicted from the DNA sequence of the structural gene.

Lipid Content of the NADH:ubiquinone Oxidoreductase Preparation

The amount of lipid present was estimated following extraction of enzyme preparations with chloroform/methanol, 2:1 (v/v) (see Experimental section for details).

Total lipid was determined gravimetrically by drying the chloroform extract to constant weight. Determinations on three separate enzyme preparations gave an average lipid content of 2.2g lipid per g protein (S.E. = 0.34) or approximately 70% by weight.

Thin layer chromatography of the chloroform/methanol

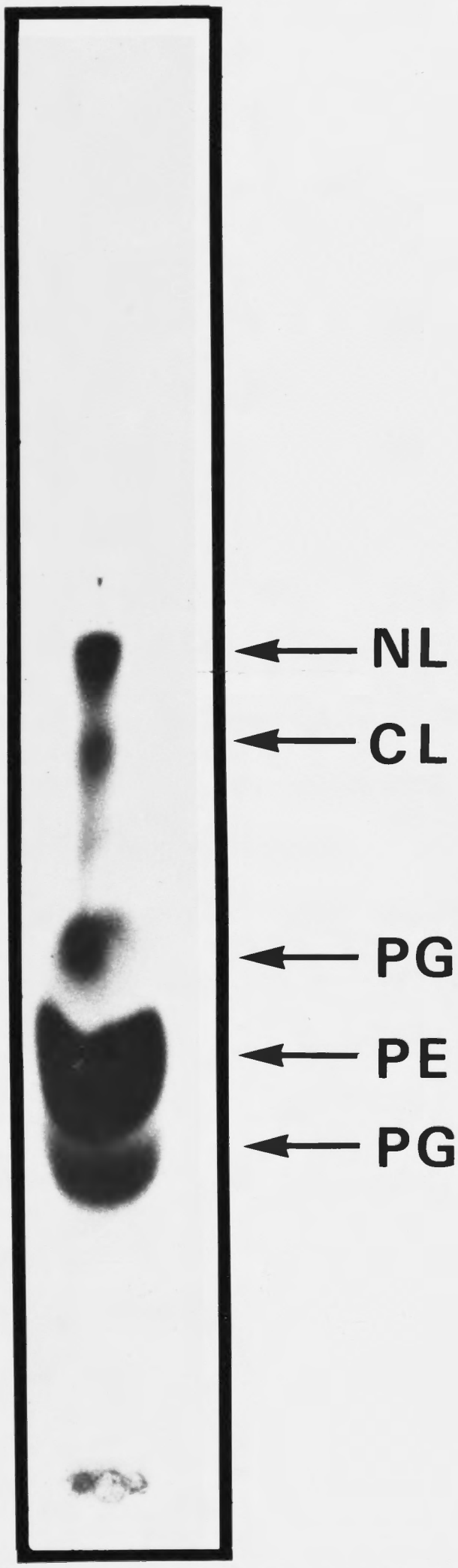
extracts on silica gel (solvent: chloroform/methanol/water, 65:31:4 (v/v)), resolved one major (R_f, 0.48) and several minor (R_f, 0.38, 0.58, 0.80, 0.90) lipid species (Figure 4-4). Comparison with pure standards, as well as the positive reaction with ninhydrin, indicated that the major lipid present is phosphatidylethanolamine. From their R_f values in this solvent system, relative to phosphatidylethanolamine, the other species correspond to phosphatidylglycerol (R_f, 0.38 & 0.58), cardiolipin (R_f, 0.80) and neutral lipid (R_f, 0.90) (Ames, 1968) but were not further identified.

The lipid extract was also assayed for phospholipid phosphorus, by the fluorometric procedure of Schiefer and Neuhoff (1971), using phosphatidylethanolamine as standard, and total phosphorus. The results of the former assay are in reasonable agreement with the dry weight determinations, and gave an estimate of 2.08g phospholipid per g protein, or 67% by weight. Total phosphorus analysis gave an estimate of 0.0852g P per g protein.

From the high lipid content of the preparation, it is likely that the enzyme has been purified as a lipoprotein complex, similar in nature to complex 1. This is expected from the known properties of the detergent used for solubilization, potassium cholate, which forms mixed micelles with lipids rather than homogeneous protein-detergent complexes.

The lipid content of complex 1, 0.22g lipid per g protein (Hatefi *et al.*, 1962) is much lower than that of the preparation described above, and this may be the result of the repeated treatment with bile salts and ammonium salts during the preparation of the former complex. There

FIGURE 4-4. Thin Layer Chromatography of Lipids Extracted from Pure Enzyme Preparations.



Solvent: chloroform/methanol/H_2O, 65:31:4 (v/v). Lipids visualized with Iodine vapour. Tentative assignments: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; NL, neutral lipid. For further details see text.

is sufficient lipid present in complex 1 to retain the rotenone-sensitive activity with ubiquinone as electron acceptor; however, that there has been a change at the active site of the complex during its isolation is shown by the 5 to 6-fold increase in the apparent K_m for ubiquinone-1 compared to the membrane-bound enzyme (44μM compared with 8μM (Hatefi & Stempel, 1969; Schatz & Racker, 1966)). Preliminary experiments with the pure *E. coli* NADH:ubiquinone oxidoreductase suggest that the apparent K_m for ubiquinone-1 (under the assay conditions described above) is approximately 1μM (data not shown).

A complete characterization of the detergent-lipoprotein complex has not been attempted, and a detailed hydrodynamic study, such as that by Dooijewaard *et al.* (1978) of complex 1 would need to be attempted. The type of data that one could derive from such a study would serve to characterize the preparation but could not be used to infer the structure of the enzyme complex in the membrane. How much lipid in the preparation is tightly associated with the enzyme is not known, and it may be that much of the lipid simply co-chromatographs with the complex.

Identification and Quantification of the Flavin Prosthetic Group of NADH:ubiquinone Oxidoreductase

During chromatography of the solubilized NADH:ubiquinone oxidoreductase on hydroxylapatite, 20μM FAD was included in the equilibration and gradient buffers. It was found that if flavin is omitted from the column buffers, the resulting preparation is inactive unless FAD is present in the assay. The reactivation of the apoenzyme by flavin was found to be rapid, and the activity obtainable could not be

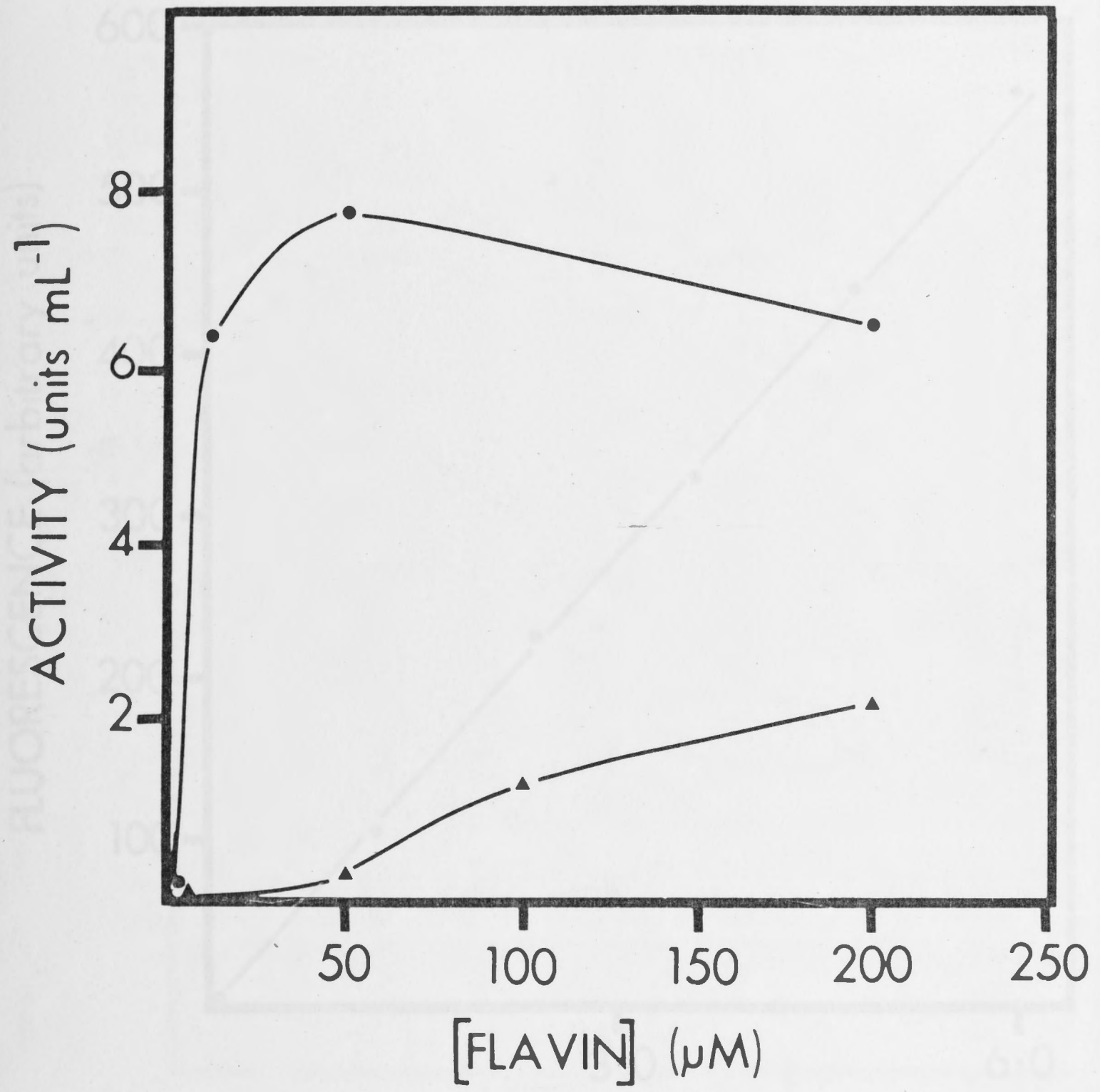
increased by preincubation with FAD for periods as long as 5 min. Maximum reactivation was obtained at approximately 50μM FAD, with higher concentrations being slightly inhibitory; the requirement for flavin was not satisfied by FMN at comparable concentrations (Figure 4-5).

The above experiments suggest that the prosthetic group on the enzyme is non-covalently-bound FAD. No covalently-bound flavin was detected when preparations of pure enzyme were electrophoresed on SDS polyacrylamide gels and analyzed fluorometrically and at acid pH by the method of Sato *et al.* (1977): 9μg of pure enzyme was electrophoresed in each analysis.

The stoichiometry of the flavin prosthetic group was estimated following dialysis of the enzyme preparation against 5mM potassium phosphate, pH7.5, 0.1% (w/v) potassium cholate, to remove flavin present in the elution buffer. Total flavin was estimated fluorometrically by the procedure of Bessey *et al.* (1949) and the values obtained from the concentration of flavin in the preparation were corrected for the determined concentration of flavin in the dialyzate. The true protein concentration of the solution was obtained by using the correction factor for Lowry protein estimation as determined in Chapter 5. A value of 1.02 ± 0.11 moles flavin per mole subunit was obtained (Figure 4-6). Assuming that no flavin has been lost from the enzyme during dialysis, this data indicates that there is one molecule of non-covalently-bound FAD per subunit.

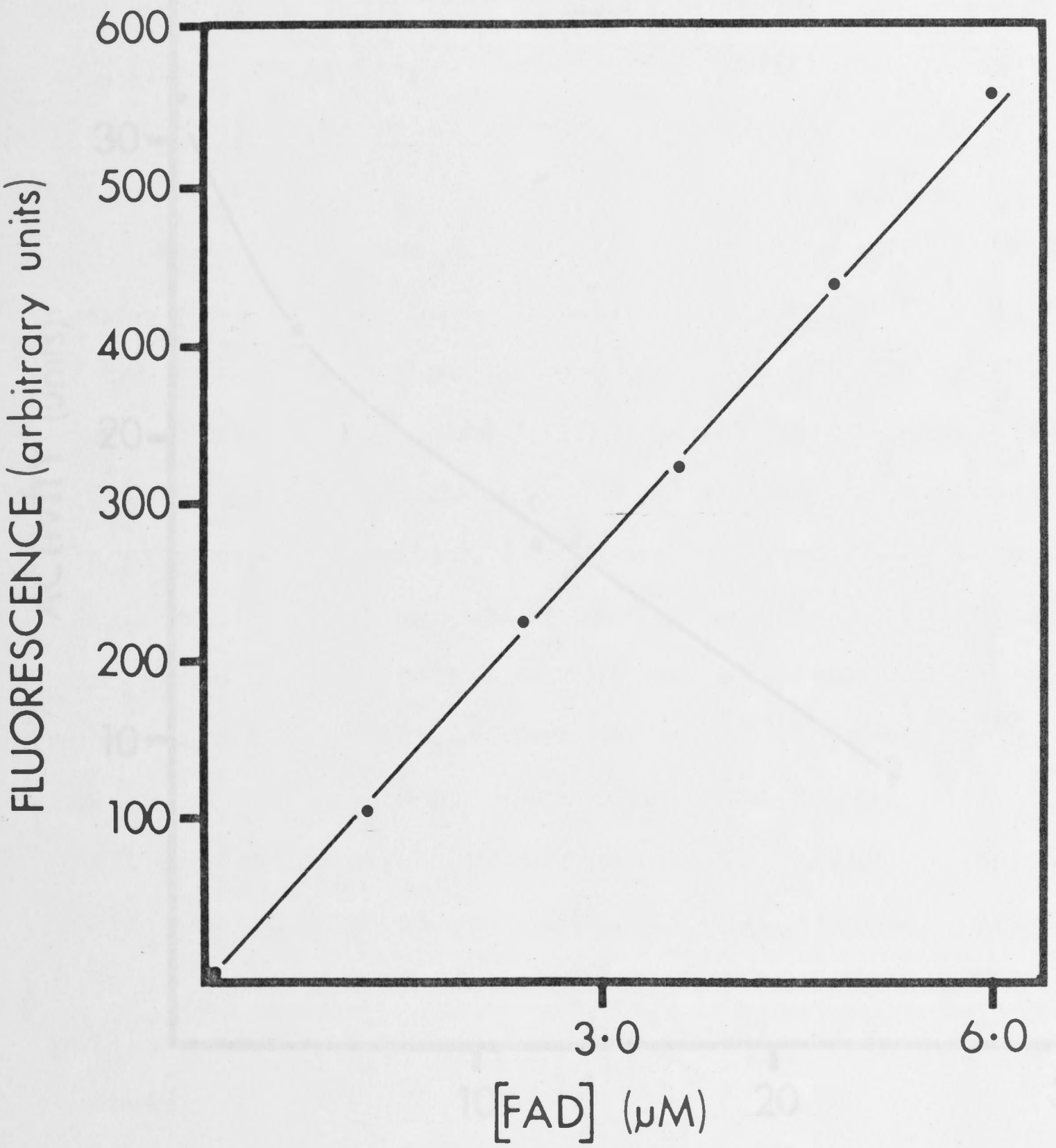
Dialysis under these conditions can sometimes lead to a gradual loss of activity, for reasons which are not fully understood (Figure 4-7). The rate of NADH oxidation,

FIGURE 4-5. Reactivation of 'De-flavo' Apoenzyme by FAD and FMN.



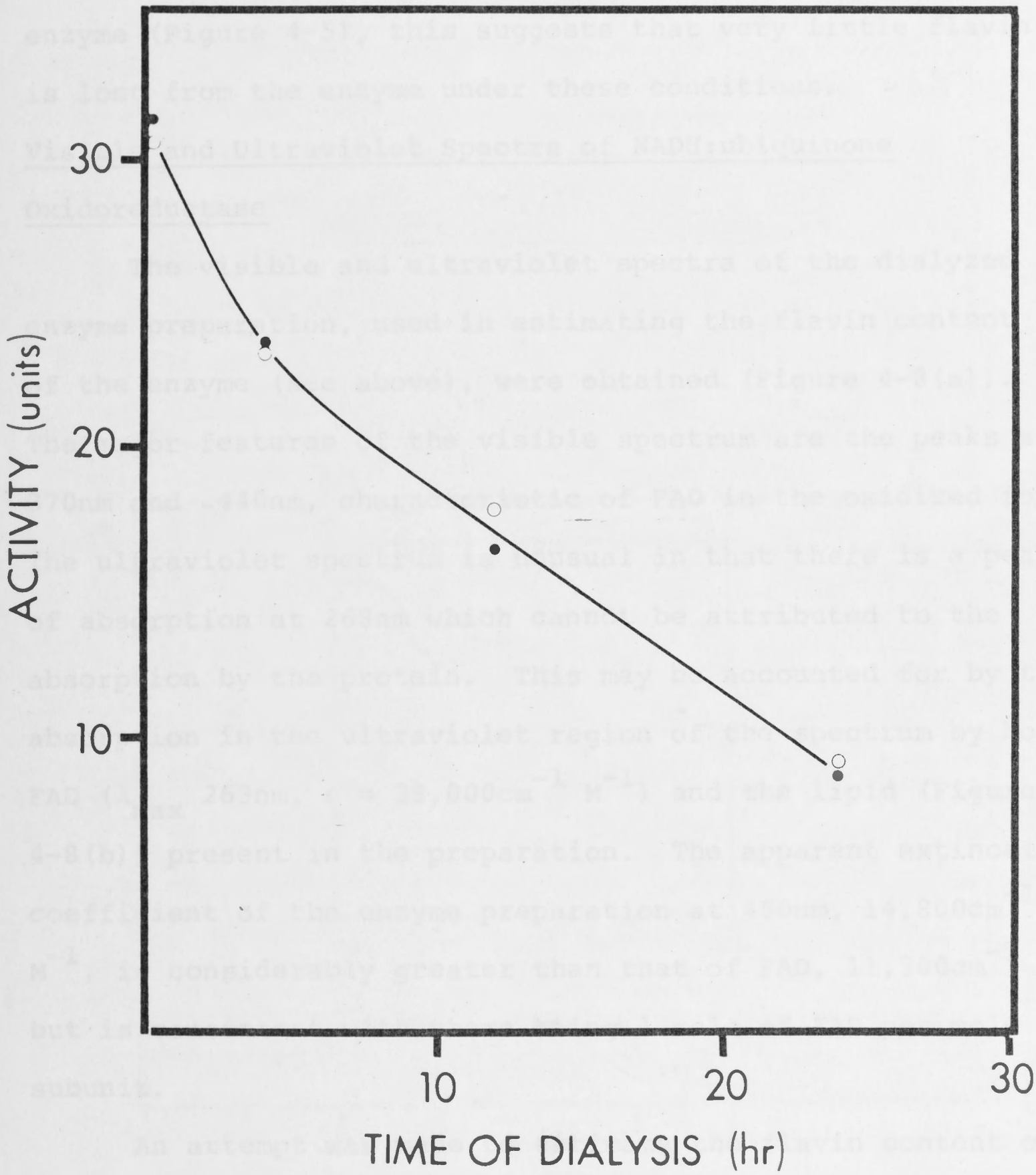
NADH:ubiquinone oxidoreductase was purified from chloramphenicol-amplified IY35 cultures exactly as described (see Chapter 2) except that FAD was omitted from the hydroxylapatite column equilibration and gradient buffers. The pooled enzyme peak was assayed for NADH: ubiquinone oxidoreductase activity under the standard assay conditions (see Chapter 2) except that 40μM FAD was replaced by either FAD or FMN at the indicated concentrations. The activity assayed in the absence of flavin was 0.027 units mL^{-1}, or 0.34% of the maximal activity obtained (at 50μM FAD); the latter, 7.82 units mL^{-1}, corresponded to a specific activity of 130 units mg^{-1}.
● —— ● FAD, ▲ —— ▲ FMN.

FIGURE 4-6. Fluorometric Determination of Total Flavin.



NADH:ubiquinone oxidoreductase was dialyzed against 5mM potassium phosphate, pH7.5, 0.1% (w/v) cholate, as described in the Experimental section. Acid-extractable flavin was determined on 0.3mL aliquots of the dialyzed enzyme preparation (concentration = 3.56μM) in triplicate, and the final dialyzate. Values were determined of 4.09, 3.50 and 3.52μM (average = 3.70μM) and 0.08μM respectively. From this data a Flavin/Enzyme stoichiometry of 1.02 (per subunit of molecular weight 47,300) was determined.

FIGURE 4-7. Loss of Activity of NADH:ubiquinone Oxidoreductase During Dialysis.



4 x 1.00mL aliquots of NADH:ubiquinone oxidoreductase (~31 units mL^{-1}) were dialyzed against 0.5L of 5mM potassium phosphate, pH7.5, 0.1% (w/v) cholate, at 4°. Dialysis buffer was changed after 4, 8 and 12h. Samples were assayed at 0, 4, 12 and 24h for NADH:ubiquinone oxidoreductase activity both under the standard assay conditions (see Chapter 2) and with the omission of FAD from the assay buffer. Activities are corrected for the volume changes after dialysis (estimated gravimetrically) and are normalized to the 0h figure.

●——● + FAD; ○——○ - FAD.

however, is not affected by the omission of FAD from the assay buffer, and since it has already been shown that FAD can rapidly reconstitute the holoenzyme from the 'de-flavo' enzyme (Figure 4-5), this suggests that very little flavin is lost from the enzyme under these conditions.

Visible and Ultraviolet Spectra of NADH:ubiquinone Oxidoreductase

The visible and ultraviolet spectra of the dialyzed enzyme preparation, used in estimating the flavin content of the enzyme (see above), were obtained (Figure 4-8(a)). The major features of the visible spectrum are the peaks at 370nm and ~440nm, characteristic of FAD in the oxidized form. The ultraviolet spectrum is unusual in that there is a peak of absorption at 268nm which cannot be attributed to the absorption by the protein. This may be accounted for by the absorption in the ultraviolet region of the spectrum by both FAD (λ_{max} 263nm, ε = 38,000cm^{-1} M^{-1}) and the lipid (Figure 4-8(b)) present in the preparation. The apparent extinction coefficient of the enzyme preparation at 450nm, 14,800cm^{-1} M^{-1}, is considerably greater than that of FAD, 11,300cm^{-1} M^{-1}, but is consistent with there being 1 mole of FAD per mole subunit.

An attempt was made to estimate the flavin content of the preparation by making a correction for the light scattering of the sample in the following manner: a plot of the logarithm of absorbance versus wavelength was found to be linear in the region 530 to 700nm, where there is no contribution by either flavin or lipid to absorbance. The contribution of light scattering to the absorbance at 450nm was then estimated by extrapolation, and subtracted to obtain

FIGURE 4-8(a). Visible and Ultraviolet Spectra of NADH:ubiquinone Oxidoreductase.

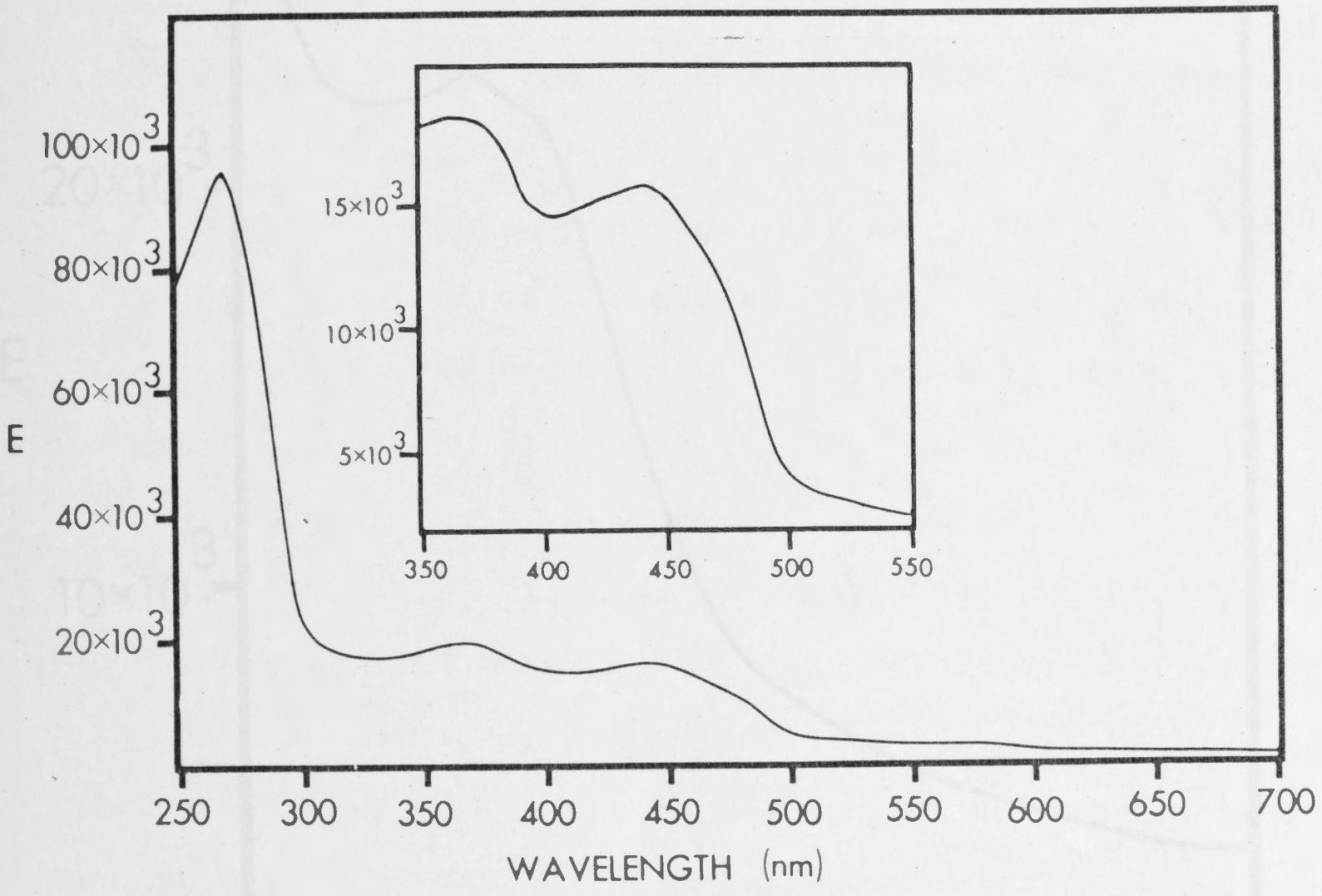
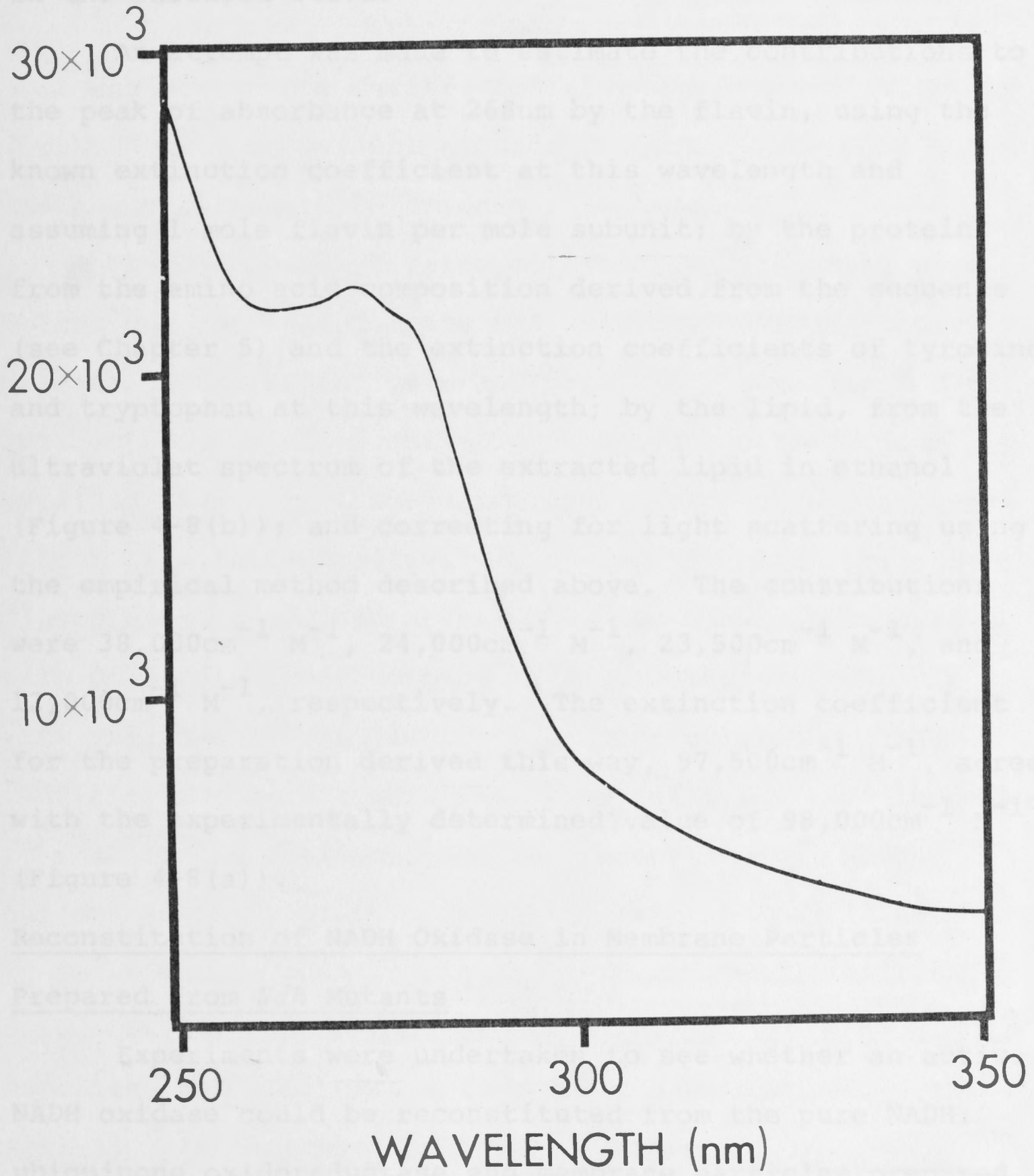


FIGURE 4-8(b). Ultraviolet Spectrum of Lipid Extracted from NADH:ubiquinone Oxidoreductase.



the corrected extinction coefficient. From this value, 11,050cm^{-1} M^{-1}, the flavin content of the preparation was estimated to be 0.98 moles per mole subunit. If we assume that the empirical method used in correcting the extinction coefficient is valid, then this indicates that there is one molecule of flavin per subunit, in agreement with the fluorometric assay, and that all of the bound flavin is in the oxidized state.

An attempt was made to estimate the contributions to the peak of absorbance at 268nm by the flavin, using the known extinction coefficient at this wavelength and assuming 1 mole flavin per mole subunit; by the protein, from the amino acid composition derived from the sequence (see Chapter 5) and the extinction coefficients of tyrosine and tryptophan at this wavelength; by the lipid, from the ultraviolet spectrum of the extracted lipid in ethanol (Figure 4-8(b)); and correcting for light scattering using the empirical method described above. The contributions were 38,000cm^{-1} M^{-1}, 24,000cm^{-1} M^{-1}, 23,500cm^{-1} M^{-1}, and 12,000cm^{-1} M^{-1}, respectively. The extinction coefficient for the preparation derived this way, 97,500cm^{-1} M^{-1}, agrees with the experimentally determined value of 98,000cm^{-1} M^{-1} (Figure 4-8(a)).

Reconstitution of NADH Oxidase in Membrane Particles Prepared from *Ndh* Mutants

Experiments were undertaken to see whether an active NADH oxidase could be reconstituted from the pure NADH: ubiquinone oxidoreductase and membrane particles prepared from *ndh* mutant strains.

IY12 membrane particles were titrated with the enzyme

preparation and assayed, spectrophotometrically, for NADH oxidase activity. This was done two ways (see Experimental section for details):

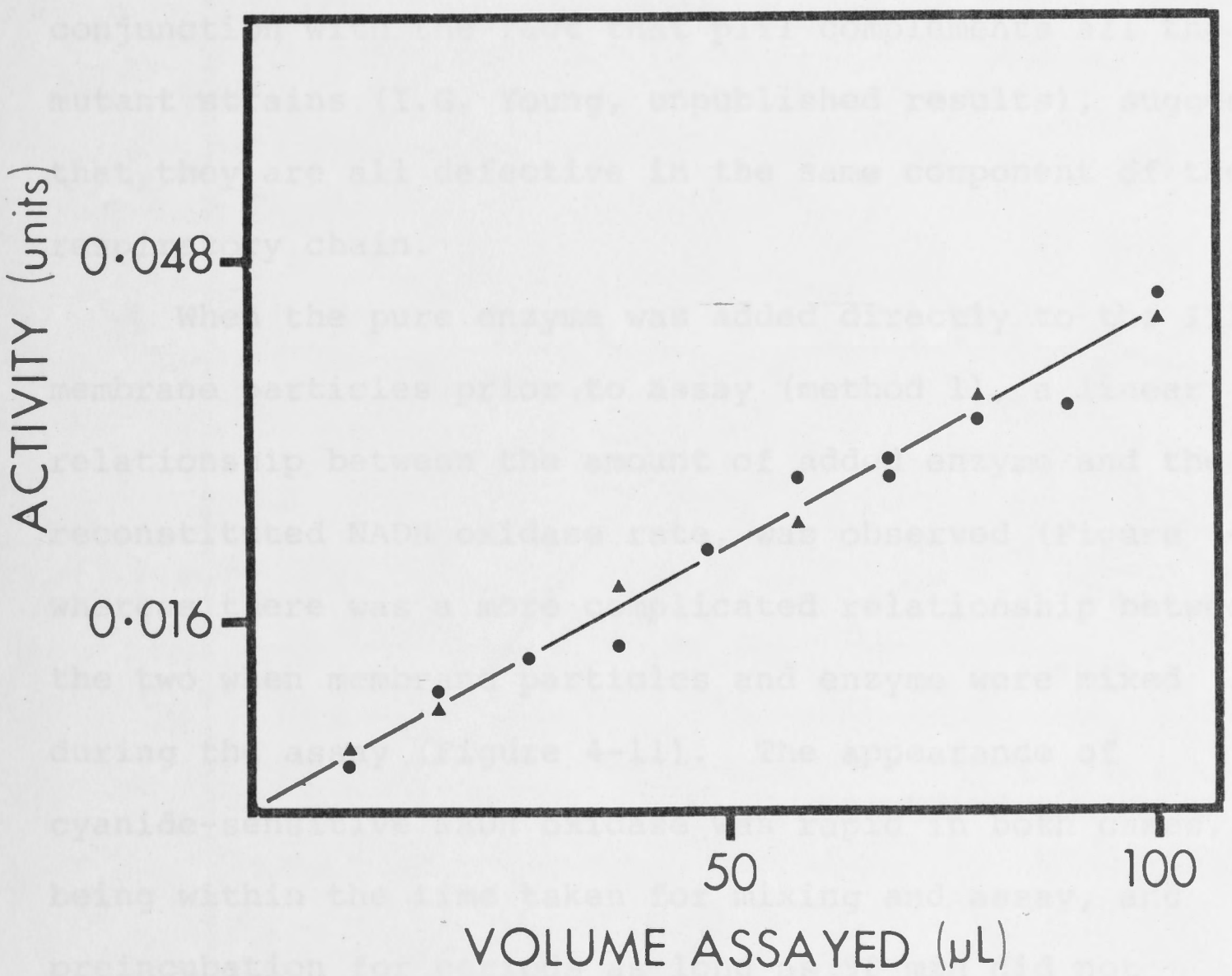
(1) Varying amounts of pure enzyme (0 to 100µL aliquots) were added to a fixed volume (0.05mL) of membrane particles at 4^{o}. THe resulting mixture was assayed for NADH oxidase.

(2) A fixed volume of membrane particles (usually 5µL) was added to a cuvette containing 1mL assay buffer and 250µM NADH, then various amounts of pure enzyme were added and the resulting NADH oxidase rate measured.

The NADH oxidase rates obtained in both cases were corrected, not only for the residual NADH oxidase activity present in the mutant membrane particles, but also for the activity of the added enzyme: it was found that the purified NADH:ubiquinone oxidoreductase possesses a cyanide-insensitive oxidase activity (Figure 4-9) characteristic of flavoprotein dehydrogenases. The NADH dehydrogenase rate with O_2 as electron acceptor is low, being less than 1% that with ubiquinone-1 as electron acceptor under the assay conditions used, but this rate is significant when compared to that of the residual, and the reconstituted, NADH oxidase activity of the *ndh* mutant membrane particles.

As can be seen in Figures 4-10 and 4-11, addition of pure NADH:ubiquinone oxidoreductase to IY12 membrane particles reconstituted a functional NADH oxidase. This activity, in contrast to those present prior to reconstitution, is sensitive to potassium cyanide (see

FIGURE 4-9. NADH Oxidase Activity of NADH:ubiquinone Oxidoreductase.



NADH:ubiquinone oxidoreductase (40.3 units mL^{-1}) was assayed spectrophotometrically for NADH oxidase activity before and after dialysis against 5mM potassium phosphate, pH7.5, 0.1% (w/v) cholate (100 vol.) at 4° for 3h. The NADH oxidase activity of the pure enzyme, 0.44 units mL^{-1}, was not affected by dialysis and was 1.1% of the ubiquinone reductase activity of the enzyme under these assay conditions.

▲ ——▲ dialyzed; ● ——● undialyzed.

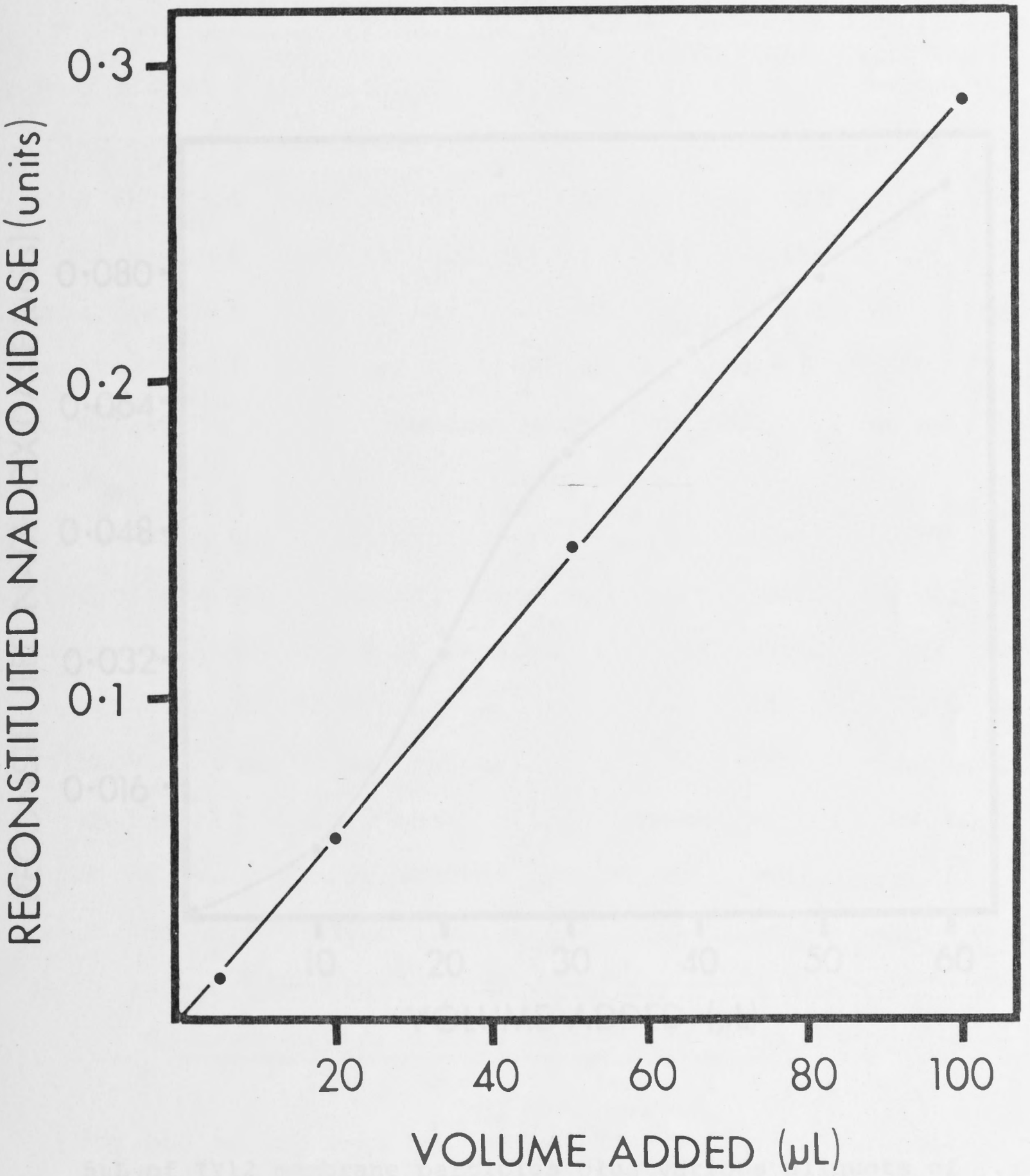
below). The specific activity of the NADH oxidase in reconstituted IY12 membrane particles approaches that of wild-type membrane particles.

The purified NADH:ubiquinone oxidoreductase could reconstitute a cyanide sensitive NADH oxidase in membrane particles derived from each of the dozen *ndh* mutant strains isolated so far (data not shown) and this, taken in conjunction with the fact that pIY1 complements all the *ndh* mutant strains (I.G. Young, unpublished results), suggests that they are all defective in the same component of the respiratory chain.

When the pure enzyme was added directly to the IY12 membrane particles prior to assay (method 1), a linear relationship between the amount of added enzyme and the reconstituted NADH oxidase rate, was observed (Figure 4-10), whereas there was a more complicated relationship between the two when membrane particles and enzyme were mixed during the assay (Figure 4-11). The appearance of cyanide-sensitive NADH oxidase was rapid in both cases, being within the time taken for mixing and assay, and preincubation for periods as long as 90 min did not increase the rate obtained: this did not hold, however, in method 1 where more than 200µL of enzyme was added to 0.05mL of particles.

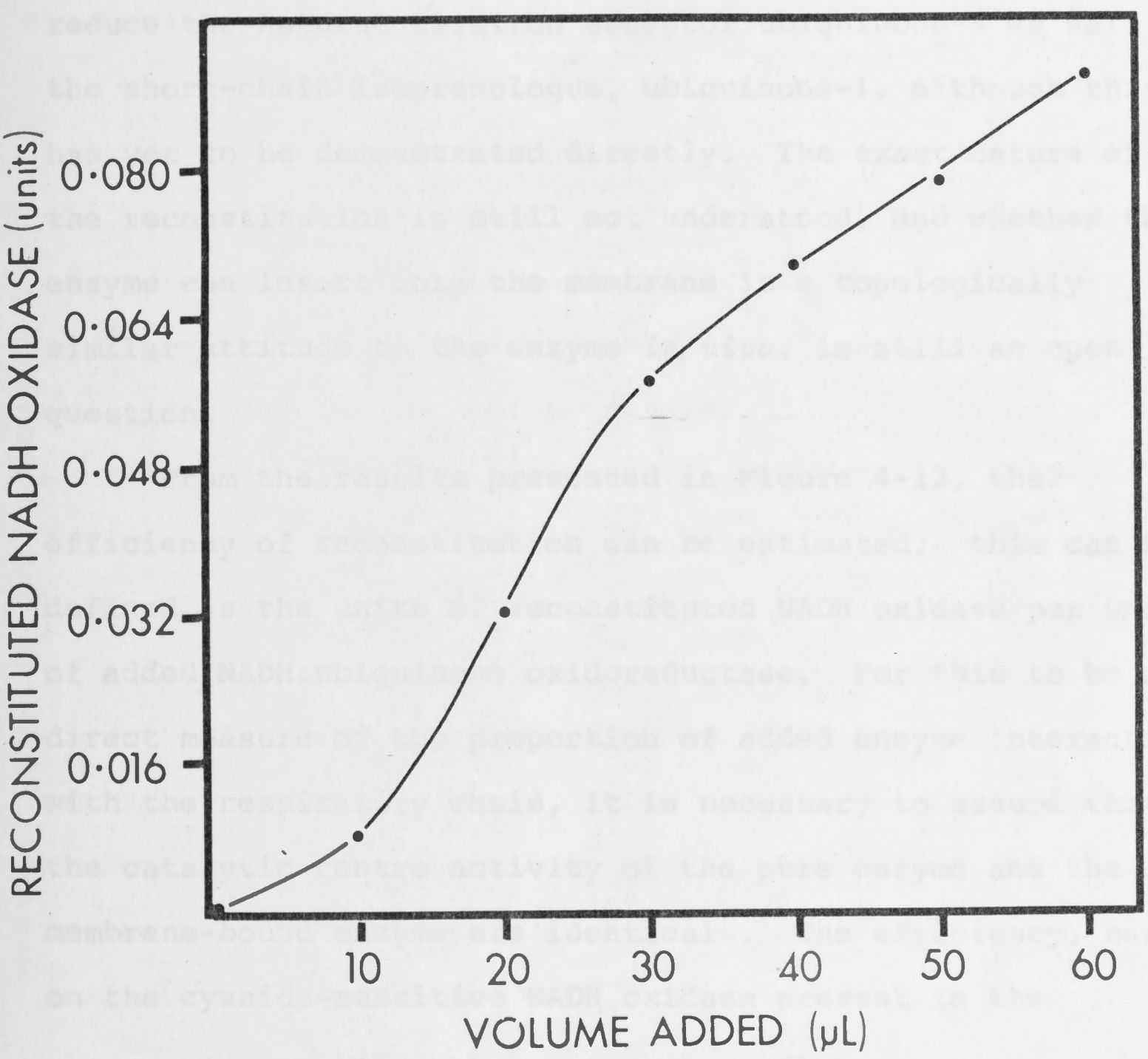
Reconstituted particles were washed in STM buffer, and the membranes collected by centrifugation (Figure 4-12). 100% of the cyanide-sensitive, and over 93% of the total, NADH oxidase activity appeared in the particulate fraction.

FIGURE 4-10. Reconstitution of NADH Oxidase in IY12 Membrane Particles: Method 1.



Various volumes of NADH:ubiquinone oxidoreductase (0 to 100µL, 24.6 units mL^{-1}) were added to 0.5mL of IY12 membrane particles, at 0 to 4°. The reconstituted particles were assayed spectrophotometrically for NADH oxidase and the rate obtained corrected for the activity of the pure enzyme (0.163 units mL^{-1}) and IY12 membrane particles (0.524 units mL^{-1}). The highest activity corresponds to a specific activity of 0.14 units mg^{-1} for the reconstituted NADH oxidase.

FIGURE 4-11. Reconstitution of NADH oxidase in IY12 Membrane Particles: Method 2



5µL of IY12 membrane particles plus various aliquots of NADH:ubiquinone oxidoreductase (0 to 60µL, 40.2 units mL^{-1}) were mixed together in 1mL assay buffer and assayed spectrophotometrically for NADH oxidase. The resulting activity was corrected for the NADH oxidase activity of the membrane particles (0.40 units mL^{-1}) and the pure enzyme (0.44 units mL^{-1}). The highest net activity measured above, corresponds to a specific activity of 0.42 units mg^{-1}.

This suggests that a proportion of added enzyme molecules has become attached to the membrane and can pass electrons from NADH to the respiratory chain, presumably at the level of the endogenous ubiquinone since no exogenous ubiquinone was added: this, in turn, implies that the pure enzyme can reduce the natural electron acceptor ubiquinone-8 as well as the short-chain isoprenologue, ubiquinone-1, although this has yet to be demonstrated directly. The exact nature of the reconstitution is still not understood, and whether the enzyme can insert into the membrane in a topologically similar attitude to the enzyme *in vivo*, is still an open question.

From the results presented in Figure 4-12, the efficiency of reconstitution can be estimated: this can be defined as the units of reconstituted NADH oxidase per unit of added NADH:ubiquinone oxidoreductase. For this to be a direct measure of the proportion of added enzyme interacting with the respiratory chain, it is necessary to assume that the catalytic centre activity of the pure enzyme and the membrane-bound enzyme are identical*. The efficiency, based on the cyanide-sensitive NADH oxidase present in the

---

* For the membrane-bound enzyme, the rate of the reaction with saturating concentrations of ubiquinone-1 as electron acceptor is not substantially different to that with the endogenous ubiquinone. This can be inferred from the fact that in wild-type membrane particles, where the respiratory NADH dehydrogenase is rate-limiting to the overall NADH oxidase rate, the rate of NADH dependent ubiquinone-1 reduction closely parallels that of the NADH oxidase.

particulate fraction after centrifugation (Figure 4-12), is 20%, and slightly higher (22%) if based on the net NADH oxidase in the pellet after correction for the activity present prior to reconstitution. Even making allowance for the assumption made, it is evident that the reconstitution is remarkably efficient.

From the NADH:ubiquinone oxidoreductase activity of the particulate and soluble fractions, and the inherent activity of the *ndh* membrane particles, the proportion of the added enzyme which becomes attached to, or inserted into, the membrane can be calculated. This figure, 3.16 units or 54.5% (allowing for the incomplete recovery of activity after washing and centrifugation) can be used to estimate the efficiency of reconstitution of the enzyme in the membrane, which can be defined as the number of units of NADH oxidase reconstituted per unit of NADH:ubiquinone oxidoreductase inserted into, or attached to, the membrane particles. This turns out to be 44%, based on the cyanide-sensitive NADH oxidase present in the washed particles, and 49% when based on the net NADH oxidase in the pellet after correction for the activity prior to reconstitution.

It was observed that the purified enzyme preparation is reconstitutively inactive after dialysis against low ionic strength buffer (Figure 4-13). This phenomenon is of interest since it is possible that some component of the enzyme, necessary for electron transfer to endogenous ubiquinone but not for ubiquinone-1 reduction, is being lost during dialysis. The significance of this is heightened by reports that short-chain analogues of ubiquinone can interact with mitochondrial membrane particles and solubilized

FIGURE 4-12. Distribution of Activities Following Washing of Reconstituted IY12 Membrane Particles

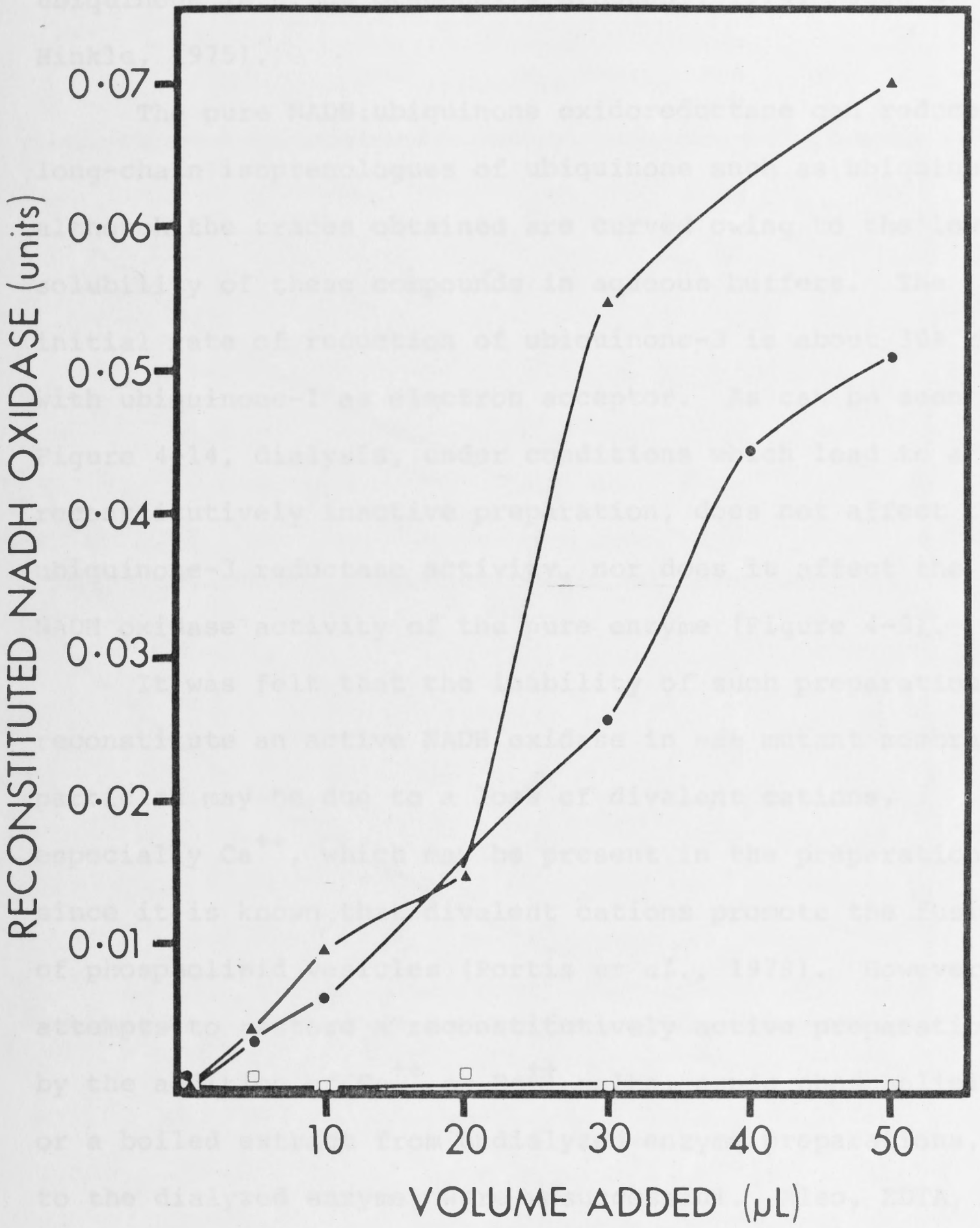
	Before Reconstitution		
	IY12 membranes	pure enzyme	total
NADH oxidase	0.16 units	0.04 units	0.20 units
CN^- sensitive NADH oxidase	0.00 "	0.00 "	0.00 "
NADH:ubiquinone oxidoreductase	0.91 "	7.12 "	8.03 "

After Reconstitution	
NADH oxidase	1.33 units
CN^- sensitive NADH oxidase	1.20 "
NADH:ubiquinone oxidoreductase	6.38 "

Membrane Particles		Supernatant	
NADH oxidase	1.63 units	NADH oxidase	0.11 units
CN^- sensitive NADH oxidase	1.40 "	CN^- sensitive NADH oxidase	0.00 "
NADH:ubiquinone oxidoreductase	4.07 "	NADH:ubiquinone oxidoreductase	2.64 "

0.5mL of IY12 membrane particles was mixed with a 0.25mL aliquot of pure NADH:ubiquinone oxidoreductase (28.5 units mL^{-1}, specific activity = 450 units mg^{-1}) at 4°. The reconstituted particles were immediately diluted with 3.45mL ice-cold STM buffer and centrifuged at 47,000 rpm for 3h in a Spinco SW56 rotor. The supernatant (vol = 4.05mL) was decanted, and the pellet resuspended in 5.0mL STM buffer. For details of assay procedures, see text. CN^- insensitive NADH oxidase was measured following the addition of 3mM KCN to the assay. 1 unit ≡ 1μmole NADH oxidised min^{-1}.

FIGURE 4-13. Reversible Loss of Reconstitution Activity of NADH:ubiquinone Oxidoreductase Activity by Dialysis.



2x1mL aliquots of NADH:ubiquinone oxidoreductase (19.4 units mL^{-1}) were dialyzed at 4° for 3h against 0.1L of 5mM potassium phosphate, pH7.5, 0.1% (w/v) cholate. After 3h, one aliquot was transferred to 0.1L of 1M potassium phosphate, pH7.5, 0.1% (w/v) cholate and dialyzed for a further 3h at 4°.
5µL of IY12 membrane particles were reconstituted with 0 to 50µL aliquots, each, of the 2 dialyzed preparations, and the undialyzed control preparation, of NADH:ubiquinone oxidoreductase by method 2 (see text). No correction was made for the small volume changes accompanying dialysis.
▲—▲ undialyzed control; □—□ dialyzed, 5mM buffer;
●—● redialyzed, 1M buffer.

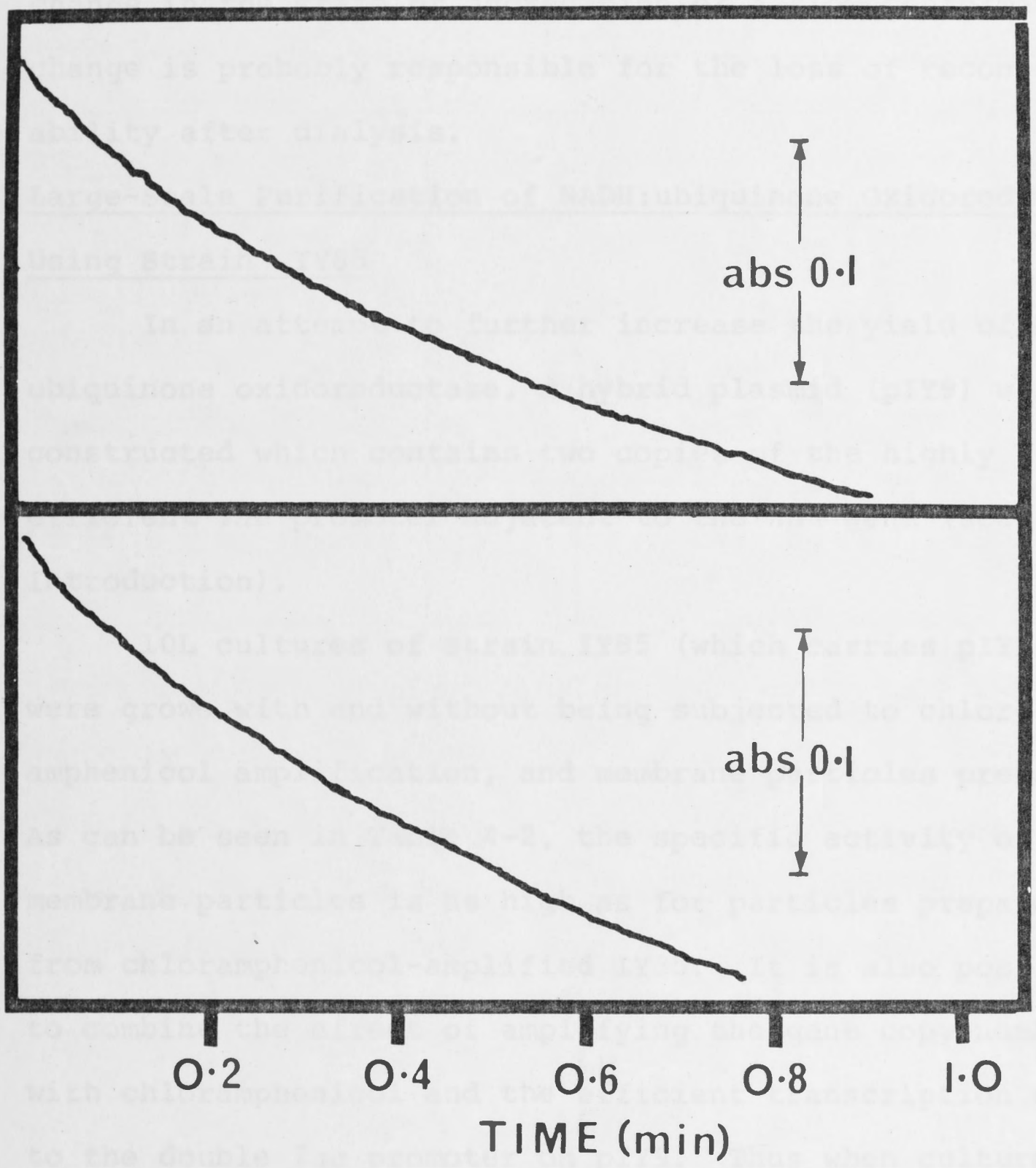
preparations at a site distinct to that of endogenous ubiquinone (*e.g.* de Otamendi & Stoppani, 1974; Ragan & Hinkle, 1975).

The pure NADH:ubiquinone oxidoreductase can reduce long-chain isoprenologues of ubiquinone such as ubiquinone-3, although the traces obtained are curved owing to the low solubility of these compounds in aqueous buffers. The initial rate of reduction of ubiquinone-3 is about 30% that with ubiquinone-1 as electron acceptor. As can be seen in Figure 4-14, dialysis, under conditions which lead to a reconstitutively inactive preparation, does not affect the ubiquinone-3 reductase activity, nor does it affect the NADH oxidase activity of the pure enzyme (Figure 4-9).

It was felt that the inability of such preparations to reconstitute an active NADH oxidase in *ndh* mutant membrane particles may be due to a loss of divalent cations, especially Ca^{++}, which may be present in the preparation, since it is known that divalent cations promote the fusion of phospholipid vesicles (Portis *et al.*, 1979). However, attempts to restore a reconstitutively active preparation by the addition of Ca^{++} or Fe^{++} salts, crude phospholipids or a boiled extract from undialyzed enzyme preparations, to the dialyzed enzyme, were unsuccessful. Also, EDTA, at a final concentration in the assay of 0.1mg mL^{-1}, was found not to inhibit the reconstitution by undialyzed enzyme preparations.

The loss of the ability to reconstitute *ndh* mutant membrane particles, after dialysis of enzyme preparations against low ionic strength buffer, is reversible, since it was found that a reconstitutively active preparation can be

FIGURE 4-14. Ubiquinone-3 Reductase Activity of NADH: ubiquinone Oxidoreductase Before and After Dialysis.



NADH:ubiquinone oxidoreductase (40.2 units mL^{-1}) was dialyzed against 100 volumes of 5mM potassium phosphate, pH7.5, 0.1% (w/v) cholate for 3h (see also Figure 4-9). Ubiquinone-3 reductase activity was assayed exactly as ubiquinone-1 reductase activity (see Chapter 2) except that ubiquinone-3 was added as electron acceptor (12.5μL of a 4mM ethanolic stock solution).

upper tracing, undialyzed enzyme;

lower tracing, dialyzed enzyme.

obtained by redialysis against 1M phosphate buffer (Figure 4-13). It is therefore unlikely that there is a loss of any component of the enzyme during dialysis. Rather, a change in the state of aggregation or a slow conformational change is probably responsible for the loss of reconstitutive ability after dialysis.

Large-Scale Purification of NADH:ubiquinone Oxidoreductase Using Strain IY85

In an attempt to further increase the yield of NADH: ubiquinone oxidoreductase, a hybrid plasmid (pIY9) was constructed which contains two copies of the highly efficient *lac* promoter adjacent to the *ndh* gene (see Introduction).

10L cultures of strain IY85 (which carries pIY9) were grown with and without being subjected to chloramphenicol amplification, and membrane particles prepared. As can be seen in Table 4-2, the specific activity of IY85 membrane particles is as high as for particles prepared from chloramphenicol-amplified IY35. It is also possible to combine the effect of amplifying the gene copy-number with chloramphenicol and the efficient transcription due to the double *lac* promoter on pIY9. Thus when cultures of IY85 were amplified with chloramphenicol, a further doubling of the specific activity occurred, giving an amplification of 80 to 100-fold relative to the level in wild-type membranes, with a small but variable amount of activity appearing in the cytoplasm.

It was hoped that the high specific activity of IY85 membrane particles might obviate the need for chlor-

TABLE 4-2. NADH Oxidase and NADH:ubiquinone Oxidoreductase Activities of Membrane and Cytoplasmic Fractions of IY85 and Chloramphenicol-amplified IY85[a]

	IY85	Cap.amp.[b] IY85 Small scale	Large scale
Membrane fraction			
NADH oxidase	4.39	5.01	3.76
NADH:ubiquinone oxidoreductase	24.5	47.9	31.8
Cytoplasmic fraction			
NADH oxidase	0.097	0.276	0.347
NADH:ubiquinone oxidoreductase	1.96	2.47	5.49

[a] Details of membrane preparations and assay conditions are given in the text.

[b] Chloramphenicol-amplified: details of chloramphenicol amplification on both small scale and large scale cultures are given in the text.

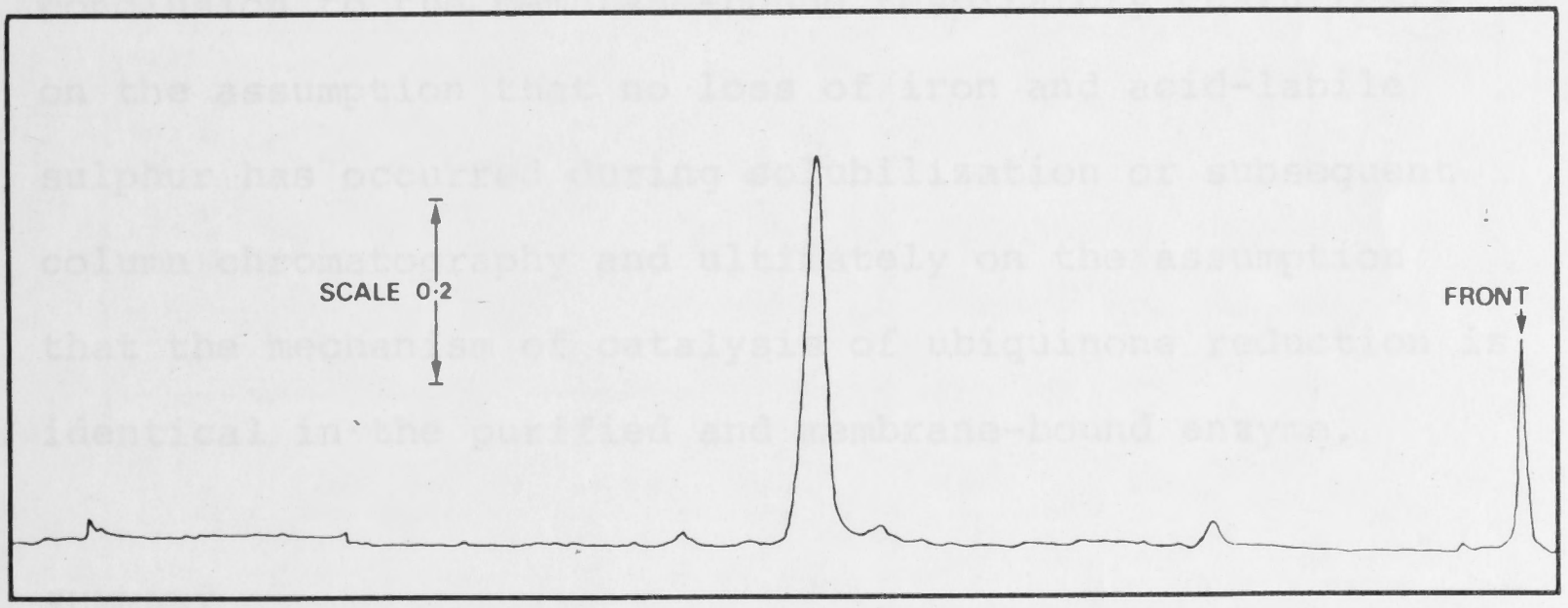
amphenicol amplification on a large-scale. Consequently, 240L of IY85 were grown in continuous culture. Unfortunately, the specific activity of membrane particles prepared from such cultures was only ~10 units mg^{-1} (Dr H.D. Campbell, unpublished results). Apparently this is a function of the continuous culture mode since 40L batch growth of IY85 in the continuous culture vessel resulted in membrane particles being prepared with specific activities >20 units mg^{-1} (ibid.). For large-scale growth of bacteria and high membrane specific activity, IY85 was grown in 40L batch culture in a stainless steel fermenter and amplified with chloramphenicol, with the second stage of growth being carried out in 9 x 14L glass fermenters (see Experimental section). The preparation of membranes and hydroxylapatite column chromatography are essentially scaled-up versions of the procedures already described (see Experimental section and Chapter 2).

Typically ~100mL of membrane particles were obtained from ~150g (wet weight) of bacteria, yielding 50 to 100mg of purified enzyme protein with a specific activity of 550 units mg^{-1}. The purity of such preparations, gauged by SDS polyacrylamide gel electrophoresis, was not noticeably different to the small-scale preparations (Figure 4-15).

Analysis of Metal Content of NADH:ubiquinone Oxidoreductase Preparations

NADH:ubiquinone oxidoreductase was purified from chloramphenicol-amplified IY85 on a large-scale: 130mL of membrane particles (specific activity 34.8 units mg^{-1}) were washed, solubilized and chromatographed on an hydroxyl-

FIGURE 4-15. SDS Polyacrylamide Gel Electrophoresis of NADH:ubiquinone Oxidoreductase Purified from Chloramphenicol-amplified IY85 on a Large Scale.



apatite column (5 x 32cm). Peak fractions were then assayed directly for activity, protein, non-haem iron, total iron, and acid-labile sulphur. Aliquots were also taken for analysis by SDS polyacrylamide gel electrophoresis. The results presented in Table 4-3 indicate that no stoichiometric amount of iron or acid-labile sulphur was detected in column fractions possessing full activity with ubiquinone-1 as electron acceptor. Analyses for Mo, W, Fe and Cu on pooled column fractions also proved negative.

The involvement of iron, especially in the form of iron-sulphur clusters, in electron transport between NADH and ubiquinone in the purified enzyme preparation can therefore be ruled out, although extension of this conclusion to the membrane-bound respiratory chain rests on the assumption that no loss of iron and acid-labile sulphur has occurred during solubilization or subsequent column chromatography and ultimately on the assumption that the mechanism of catalysis of ubiquinone reduction is identical in the purified and membrane-bound enzyme.

SUMMARY

Following hydroxylapatite column chromatography of cholate-solubilized membrane particles from genetically-amplified strains, an NADH:ubiquinone oxidoreductase has been purified 800 to 1000-fold relative to the activity present in wild-type membranes. The specific activity with ubiquinone-1 as electron acceptor, ~530 units mg^{-1} measured at 30^{o}, is the highest reported for any preparation. The enzyme is specific for NADH as electron donor.

Comparison with analogous column profiles of cholate-

TABLE 4-3. Activity and Iron Content of Peak Fractions during Large-scale Hydroxylapatite Column Chromatography

Fraction	Activity (Units mL^{-1})	Protein (mg mL^{-1})	Specific Activity (Units mg^{-1})	[Protein] (μM)	[Total Fe] (μM)	Fe/Protein
132	10.8	0.190	57	3.45	0.322	0.09
136	42.8	0.192	222	3.49	0.645	0.18
140	92.6	0.203	456	3.69	0.902	0.24
144	111.5	0.181	617	3.29	0.452	0.14
148	91.2	0.147	621	2.67	-	-
152	45.2	0.106	425	1.93	0.257	0.13

90L of chloramphenicol amplified IY85 were grown, and enzyme purified on a large scale. 130mL of membrane particles (specific activity 34.8 units mg^{-1}) were solubilized and chromatographed on a column of hydroxylapatite (5 x 32cm) as described in the text. 25mL fractions were collected: the peak tubes of activity were assayed for NADH:ubiquinone oxidoreductase, protein, total iron, and non-haem iron (see Experimental section).

Protein concentration was determined from the Lowry estimate using MW 47304 and as described in Chapter 5.

solubilized membrane particles prepared from strains IY12 and IY13, indicate that the pure enzyme is the respiratory NADH dehydrogenase and may represent the respiratory NADH: ubiquinone oxidoreductase in a highly purified form.

The enzyme consists of a single subunit type of apparent molecular weight 44,600, estimated by SDS polyacrylamide gel electrophoresis, and one molecule of non-covalently bound flavin per subunit. Reconstitution studies with 'de-flavo' apoenzyme strongly suggest that the flavin prosthetic group is FAD. The preparation contains 2.2g lipid per g protein but no Fe, acid-labile S, Mo, W or Cu. There is no evidence for other prosthetic groups from the visible or ultraviolet spectra of the pure enzyme.

The preparation is reconstitutively active: a cyanide-sensitive NADH oxidase can be reconstituted by mixing pure enzyme and *ndh* mutant membrane particles. This process is very efficient, and nearly 50% of the pure enzyme which becomes attached to the membrane particles can participate in electron transport via the endogenous ubiquinone.

CHAPTER 5.

Protein Chemical Studies on the Respiratory NADH dehydrogenase

INTRODUCTION

In previous chapters the identification and purification of the respiratory NADH:ubiquinone oxidoreductase from *E. coli* membrane particles has been presented. As well as defining the role of this enzyme in respiration, our interest is in developing the preparation as a model system to study the basic properties of membrane proteins and, ultimately, membrane-bound enzymes.

One of the basic pieces of information, which was accessible following the cloning of the structural gene and the subsequent purification of the enzyme, is the complete primary structure of the polypeptide chain. This information is not only necessary in any future mechanistic study on the enzyme, but is also intrinsically important in view of the fact that very little sequence data is available for enzymes which are intrinsic membrane proteins.

Of the two approaches possible (*viz.* protein and DNA sequencing), it was felt that DNA sequencing is the more powerful technique, and was chosen to obtain the requisite data. The protein chemical studies which are described in this chapter, were initiated with the following objectives in mind:

1. To confirm the single subunit nature of the enzyme.

2. To obtain sufficient data to check the accuracy of the nucleic acid sequence and to confirm the identification of the structural gene within the cloned DNA fragment.

3. To lay the groundwork for future structural studies.

The first two objectives are self-explanatory. With respect to the third, experiments are being planned to map the active site of the enzyme with photoaffinity substrate analogues, and the topology of the membrane-bound enzyme with surface labelling reagents. Identification of the reactive residues can be accomplished following isolation of the relevant tryptic peptides containing such a residue, and assignment of that peptide in the primary sequence.

The DNA sequence of the structural gene has resulted in an unexpected discovery related to the initiation of protein synthesis, in that the structural gene possesses a unique and novel initiation codon (B.L. Rogers, H.D. Campbell, & I.G. Young, unpublished results. See below). The importance of this finding has led to further protein chemical studies aimed at positively identifying the amino terminal sequence of the plasmid-coded gene product.

The work presented in this chapter was done in close co-operation with Dr D.C. Shaw, who also operated the protein sequencer. I also thank Dr I.G. Young for his permission in reproducing the nucleic acid and derived amino acid sequences.

EXPERIMENTAL

Amino Acid Composition of NADH:ubiquinone Oxidoreductase

NADH:ubiquinone oxidoreductase was purified on a large scale from chloramphenicol-amplified IY85 cultures as described in Chapter 4. Active fractions were pooled and dialyzed at 4^{o} against 3 x 40 volumes of 5mM potassium phosphate buffer, pH7.5, and 2 x 40 volumes of glass distilled water, with 10 to 12h between each buffer change. After dialysis, 1mL aliquots were accurately transferred to

glass hydrolysis tubes and an equal volume of conc. HCl was added. The tubes were evacuated, sealed, and heated at 110° for 22, 48 and 96h, then the hydrolyzate was analyzed on a modified Beckman 120C amino acid analyzer, fitted with a single 6mm column of W2 resin (Beckman Instruments Inc., Palo Alto, Calif.). Peaks were integrated with a Beckman 126 Data System.

1mL aliquots of the dialyzed enzyme preparation were taken for simultaneous protein estimation using the procedure of Lowry *et al.* (1951) (see Chapter 2), and the remainder freeze dried.

Delipidation of the freeze-dried material was carried out at 4°: 53mg of solid was transferred to a 15mL glass centrifuge tube and 1mL of chloroform/methanol, 2:1 (v/v) (chilled to -15°) was added, and the mixture vortexed for 1 min. A second 1mL of solvent was added and the mixture again vortexed for 1 min. A further 2mL of solvent was added and, after a short mix, the protein pelleted by centrifugation (10 min., 2,000 rpm). The extraction was repeated 5 times. After the last extraction, the protein pellet was dried under N_2, washed twice in ice-cold distilled water and desiccated under vacuum.

This material, with and without performic acid oxidation (Hirs, 1956), was analyzed following hydrolysis at 110° in 6N HCl for 22h.

The tryptophan content was estimated as described by Penke *et al.* (1974) following mercaptoethane sulphonic acid hydrolysis of delipidated enzyme.

Dual Column Amino Acid Analysis

NADH:ubiquinone oxidoreductase was purified on a small

scale from chloramphenicol-amplified IY35 cultures as described in Chapter 4. Samples were hydrolyzed as described above then analyzed on a dual column Beckman 120C analyzer. The peaks were quantified manually.

Peptide Mapping of NADH:ubiquinone Oxidoreductase

Dialyzed and delipidated NADH:ubiquinone oxidoreductase (~6.6mg) was oxidized with performic acid (Hirs, 1956). The oxidized protein was resuspended in 4mL of 1% (w/v) ammonium bicarbonate, pH~8, then digested with 1% (w/w) (relative to the weight of oxidized protein) of TPCK treated trypsin (Worthington) for 2h at 37°. A further 70µg trypsin was added and the digestion continued for another 2h. The digested protein was then centrifuged to remove any precipitated material (pH8 core peptides). The soluble peptides were lyophilized, dissolved in 1mL H_2O, then acidified to pH4 with acetic acid. The insoluble material which precipitated on standing (pH4 core peptides) was removed by centrifugation. The amino acid composition of both the pH8 and pH4 core peptides was analyzed after hydrolysis with 6N HCl at 110° for 22h as described above.

The soluble peptides were lyophilized and redissolved in 100µL H_2O, then applied to a sheet of Whatman 3MM paper (46 x 57cm) as a band ~2cm wide). Peptides were resolved in the first dimension by electrophoresis at pH4.7 (1.5h, 40Vcm^{-1}) using the buffer system pyridine/glacial acetic acid/H_2O, 25:25:950 (v/v) in an apparatus similar to that described by Michl (1959) using mineral turpentine ("Varsol") as heat exchanger. After electrophoresis, the peptides were cut out as a strip 3.5 x 57cm and sewn onto a fresh sheet of Whatman 3MM paper. Separation in the second dimension

was by ascending paper chromatography in n-butanol/glacial acetic acid/H_2O/pyridine, 15:3:12:10 (v/v) for 16h (Waley & Watson, 1953).

Peptides were visualized by dipping the maps in a solution of ninhydrin (0.2% (w/v) in acetone).

Alternatively, maps were sprayed with a solution of fluorescamine ('Fluram', Hoffman-La Roche); 2mg L^{-1} in acetone and containing 6mL pyridine per litre, and the peptides viewed under UV light (Chromato Vue, Ultraviolet Products Inc., St. Gabriel, Calif.). The spots were circled with pencil and cut out. The peptides were extracted by macerating the cut-out spots in 6N HCl, filtered, then hydrolyzed and analyzed by amino acid analysis as described above.

When further purification was required, peptides were cut out as strips 2 x 12cm, sewn onto fresh sheets of Whatman 3MM paper, and electrophoresed in a third dimension at pH1.9 (1.5h, 40Vcm^{-1}) in the buffer system acetic acid/formic acid/H_2O, 87:22.5;890.5 (v/v), and extracted as above.

Two-dimensional peptide maps were stained specifically for tyrosine (Easley, 1965), arginine (Yamada & Itano, 1966) and histidine (Easley, 1965).

CNBr Cleavage

NADH:ubiquinone oxidoreductase was dialyzed and delipidated as described above; 5mg was then reduced and carboxymethylated by the procedure of Crestfield *et al.* (1963). CNBr cleavage was carried out as described by Gross (1967) and the more soluble of the resulting peptides separated by two-dimensional paper chromatography

(see above). Peptides were visualized with Fluram, cut out, and analyzed by amino acid analysis as described above.

Detection of Formyl-homoserine

Reduced and carboxymethylated NADH:ubiquinone oxido-reductase (~2mg) was cleaved with CNBr. The CNBr peptides were dissolved in 0.5mL of 50% (v/v) acetic acid and 80% of this material was chromatographed on a Sephadex G25 column (1.5 x 18cm), pre-equilibrated in 50% (v/v) acetic acid. The peptides were eluted with the same buffer. 0.5mL fractions were collected, and those fractions, corresponding to the elution position of free amino acids, were collected (vol. 7.5mL). The pooled fractions were rotary evaporated, resuspended in 0.5mL of 50% (v/v) acetic acid, and loaded onto a column of Dowex 50 x 2, H^+ form, (0.8 x 8cm), and eluted with ~15mL H_2O. The water eluate was hydrolyzed with 3N HCl at 110° for 22h, and then analyzed on a single column Beckman 120C amino acid analyzer.

The homoserine content was estimated from the sum total of the homoserine and homoserine lactone peaks: the former eluting before glutamic acid, and the latter between histidine and lysine on the above analysis. The concentrations of homoserine and homoserine lactone were estimated using the colour factor for aspartate and are therefore only approximations.

Determination of Amino Terminal Sequence

Reduced and carboxymethylated NADH:ubiquinone oxido-reductase (see above) was sequenced directly in a Beckman 890C spinning-cup liquid-phase sequencer, using Beckman

quadrol program 122974 for proteins. Identification of the residues at each cycle was made after hydrolysis of the corresponding PTH derivatives with 6N HCl + $SnCl_2$ (1mg mL^{-1}) for 4h at 150° (Mendez & Lai, 1975). This procedure does not allow the positive identification of tryptophan or serine, whose PTH derivatives are hydrolyzed to glycine plus alanine and alanine respectively. Similarly, distinction between aspartic acid and glutamic acid from their respective amides is not possible.

RESULTS AND DISCUSSION

Amino Acid Composition of NADH:ubiquinone Oxidoreductase

The amino acid composition of NADH:ubiquinone oxidoreductase was determined for enzyme prepared on a large scale from chloramphenicol-amplified IY85 cultures. The pure enzyme was exhaustively dialyzed, and duplicate 1mL aliquots were hydrolyzed in 6N HCl for 22, 48 and 96h. A portion of the dialyzed enzyme preparation was also freeze dried and extracted with chloroform/methanol, 2:1 (v/v) to remove lipid, and this material was analyzed after hydrolysis in 6N HCl for 22h with and without prior performic acid oxidation. The results of these analyses, shown in Table 5-1, are presented as the value for each amino acid normalized to the alanine content, and assuming 39 alanine residues per polypeptide chain (deduced from the amino acid sequence, see Table 5-1).

In general, the analyses are comparable, suggesting that the high phospholipid content does not interfere with the amino acid analysis. Oxidation, during hydrolysis of the non-performic acid-oxidized material, can be inferred

TABLE 5-1. Amino Acid Composition of NADH:ubiquinone Oxidoreductase : Large-scale Preparation

Amino acid	1	2	3	4
Lys	84.3	25.4	24.4	25
His	12.2	12.0	11.7	17
Arg	21.1	21.3	21.3	22
Asx	40.0	39.9	39.4	41
Thr	22.3[a]	20.9	21.0	26
Ser	21.4[a]	20.6	20.9	20
Glx	41.1	41.1	42.0	37
Pro	16.6	17.4	18.0	17
Gly	39.4	37.7	38.5	42
Ala	39	39	39	39
Cys	-	-	3.9[d]	4
Val	28.6[b]	26.3	25.4	27
Met	-	-	12.3	15
Ile	22.9[b]	21.4	20.9	26
Leu	46.4	46.4	46.2	51
Tyr	8.8	6.4	3.9	12
Phe	11.5	11.0	11.1	11
Trp	2.5[c]	-	-	2

NADH:ubiquinone oxidoreductase was purified on a large-scale and delipidated as described in the Experimental section.

column 1: Data averaged for duplicate 22, 48 and 96h hydrolyses on non-delipidated enzyme.

column 2: Delipidated enzyme, 22h hydrolysis.

column 3: Delipidated enzyme oxidized with performic acid, 22h hydrolysis.

column 4: Amino acid composition predicted from DNA sequence (see Chapter 5).

[a] Estimated by extrapolation to zero time.

[b] Estimated from the 96h hydrolysis values.

[c] Estimated separately by mercaptoethane sulphonic acid hydrolysis.

[d] Corrected assuming 94% recovery (Moore, 1963).

Amino acid values were normalized to the alanine content and are expressed as residues per polypeptide assuming ala = 39 (see column 4). For further details see text.

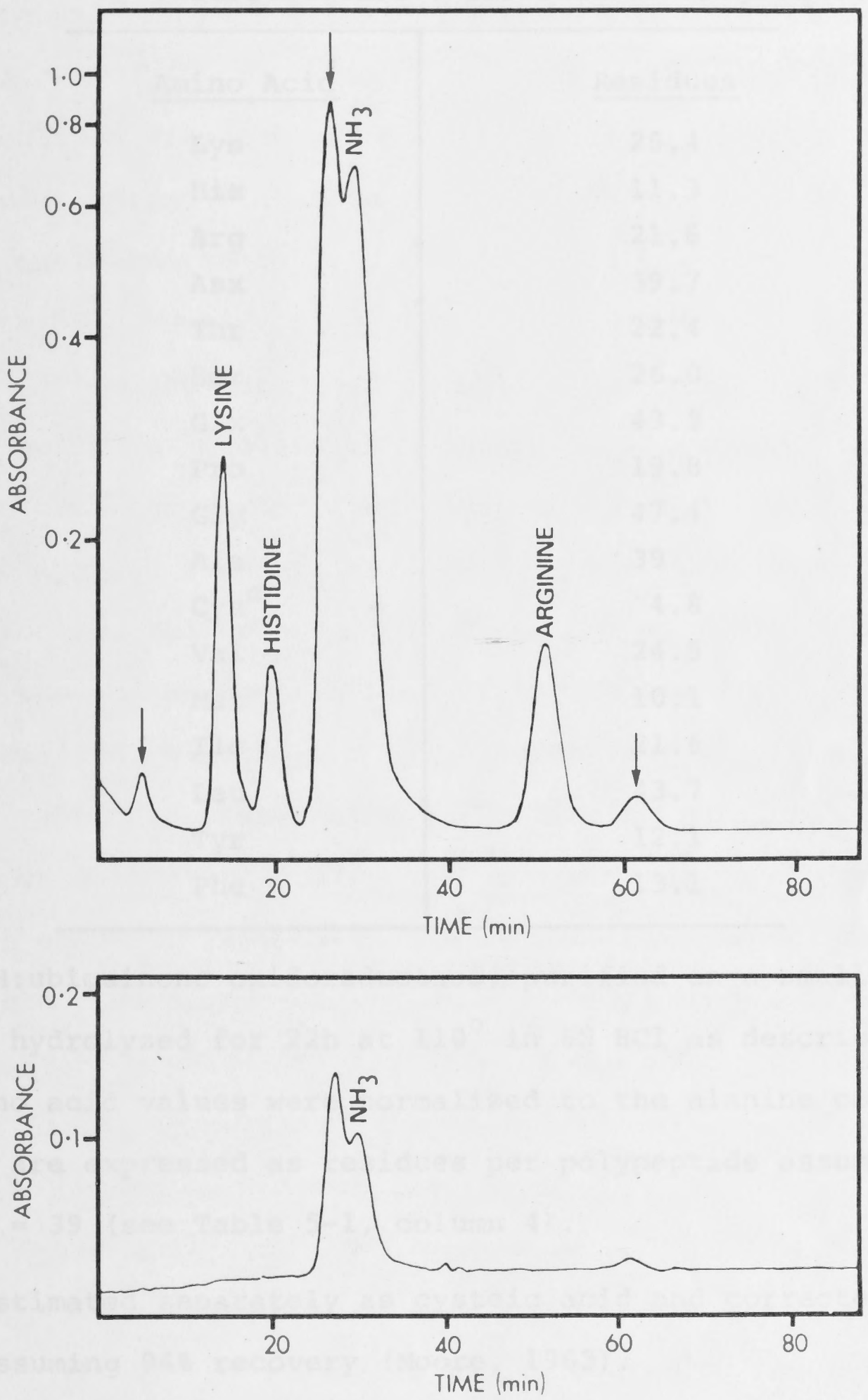
from the presence of methionine sulphoxides as well as some cysteic acid in the relevant chromatograms. This suggests that the value for tyrosine may be low and that the value for methionine should be obtained as methionine sulphone from the performic acid oxidized sample.

One major discrepancy between the analyses is in the lysine content of the preparation. The very high value obtained with the non-delipidated preparation suggests that some lipid component is interfering with the quantification of this particular amino acid. Amino acid analyses of enzyme prepared on a small scale from chloramphenicol-amplified IY35 cultures, and performed on a dual-column amino acid analyzer, suggest that an hydrolysis product of phosphatidylethanolamine co-chromatographs with lysine on the single column analyzer (Figure 5-1): during analysis of such preparations several unknown peaks were resolved on the short column of the Beckman 120C analyzer, which correspond in their elution positions to peaks obtained when pure phosphatidylethanolamine was hydrolyzed and analyzed under identical conditions (Figure 5-1).

In general, the results of the analyses of the small-scale preparations agree with those obtained on material prepared on a large scale (*c.f.* Tables 5-1 and 5-2) with the exception of serine, which is unaccountably higher in the former. In neither case, however, is there any marked difference in the serine content of preparations subject to delipidation, which suggests that, as expected (see Ames, 1968), phosphatidylserine is not present in the preparation in large amounts.

The tryptophan content of the protein was estimated

FIGURE 5-1. Dual Column Amino Acid Analysis of NADH: ubiquinone Oxidoreductase and Phosphatidyl ethanolamine



Samples of NADH:ubiquinone oxidoreductase (~200µg) and *E. coli* phosphatidylethanolamine (100µg) were hydrolyzed in 6N HCl for 22h at 110°. Aliquots of the hydrolyzates (½ and 1/5 respectively) were analyzed on a dual column Beckman 120C analyzer. Only the short-column profiles are shown; no extra peaks are visible on the long-column profiles. Positions of the unknown peaks in the enzyme analysis are shown with arrows.

Top, NADH:ubiquinone oxidoreductase; bottom, phosphatidyl-ethanolamine.

TABLE 5-2. Amino Acid Composition of NADH:ubiquinone Oxidoreductase : Two-column Analysis

Amino Acid	Residues
Lys	25.4
His	11.3
Arg	21.6
Asx	39.7
Thr	22.4
Ser	26.0
Glx	43.9
Pro	19.8
Gly	47.4
Ala	39
Cys[a]	4.8
Val	24.5
Met	10.1
Ile	21.6
Leu	43.7
Tyr	12.1
Phe	13.1

NADH:ubiquinone oxidoreductase, purified on a small-scale, was hydrolyzed for 22h at 110° in 6N HCl as described. Amino acid values were normalized to the alanine content and are expressed as residues per polypeptide assuming ala = 39 (see Table 5-1, column 4).

[a] Estimated separately as cysteic acid and corrected assuming 94% recovery (Moore, 1963).

separately following mercaptoethane sulphonic acid hydrolysis of pure enzyme prepared from chloramphenicol-amplified IY35 cultures. The tryptophan content, calculated from the amino acid values normalized to alanine (and assuming 39 alanine residues per polypeptide chain) was estimated to be 2.49 residues per polypeptide chain (data not shown).

From the data used in obtaining column 1 of Table 5-1, the weight of protein in the sample analyzed was calculated from the recovery of individual amino acids. Corrections were applied for the loss or incomplete recovery of serine, threonine, valine and isoleucine, and an estimate included for the weight contribution of tryptophan (from the normalized value to the alanine content as determined above) and methionine (estimated as methionine sulphone). A concentration of 35.14μg mL^{-1} protein was determined in this manner. From estimates of the protein concentration by the procedure of Lowry *et al.* (1951) on the same material, a value of 40.94μg mL^{-1} was obtained. This gives the following relationship:

[Protein] ≡ 'Lowry estimate' x 0.86.

The analytical data agrees with the amino acid composition obtained from the sequence of the polypeptide chain (Table 5-1), for all the stable amino acids with the exception of histidine and, to a lesser extent, leucine. The reason for the discrepancy with histidine is not understood, although hydrolytic losses of about 20% have been observed in control experiments under identical conditions (D.C. Shaw, personal communication).

Using the amino acid sequence data, the partial

specific volume was calculated by the method of Cohn & Edsall (1943) to be 0.747g cm^{-3}. Using the same data, several parameters were calculated which reflect the hydrophobicity of the polypeptide chain. The polarity index (Capaldi & Vanderkooi, 1972) was calculated to be 43.3%, whilst the average hydrophobicity, $H_{\phi av}$, is 1,061 calories $residue^{-1}$ (Bigelow, 1967). These figures show that the polypeptide chain is not significantly hydrophobic overall, relative to the polypeptide chains of soluble proteins (see above references), and that therefore the membrane-bound nature of this enzyme is a function of the specific folding of the polypeptide chain which must generate hydrophobic regions capable of interacting with phospholipid.

Peptide Mapping of the NADH:ubiquinone Oxidoreductase

NADH:ubiquinone oxidoreductase was delipidated following exhaustive dialysis, then oxidized with performic acid and digested with TPCK treated trypsin. A portion of the soluble peptides (2mg) was then resolved by two-dimensional paper chromatography on Whatman 3MM paper, and visualized by dipping the resulting tryptic map in ninhydrin. The insoluble 'pH8 core' peptides, present after the tryptic digestion, as well as the 'pH4 core' peptides, which precipitated on lowering the pH prior to electrophoresis, were collected by centrifugation and analyzed separately, after hydrolysis in 6N HCl, by amino acid analysis (Table 5-3).

A total of 39 spots were visible on the tryptic map (Figure 5-2), but there is also the possibility of there being several polypeptides present in the core material. From the arginine plus lysine content of the enzyme (22 and

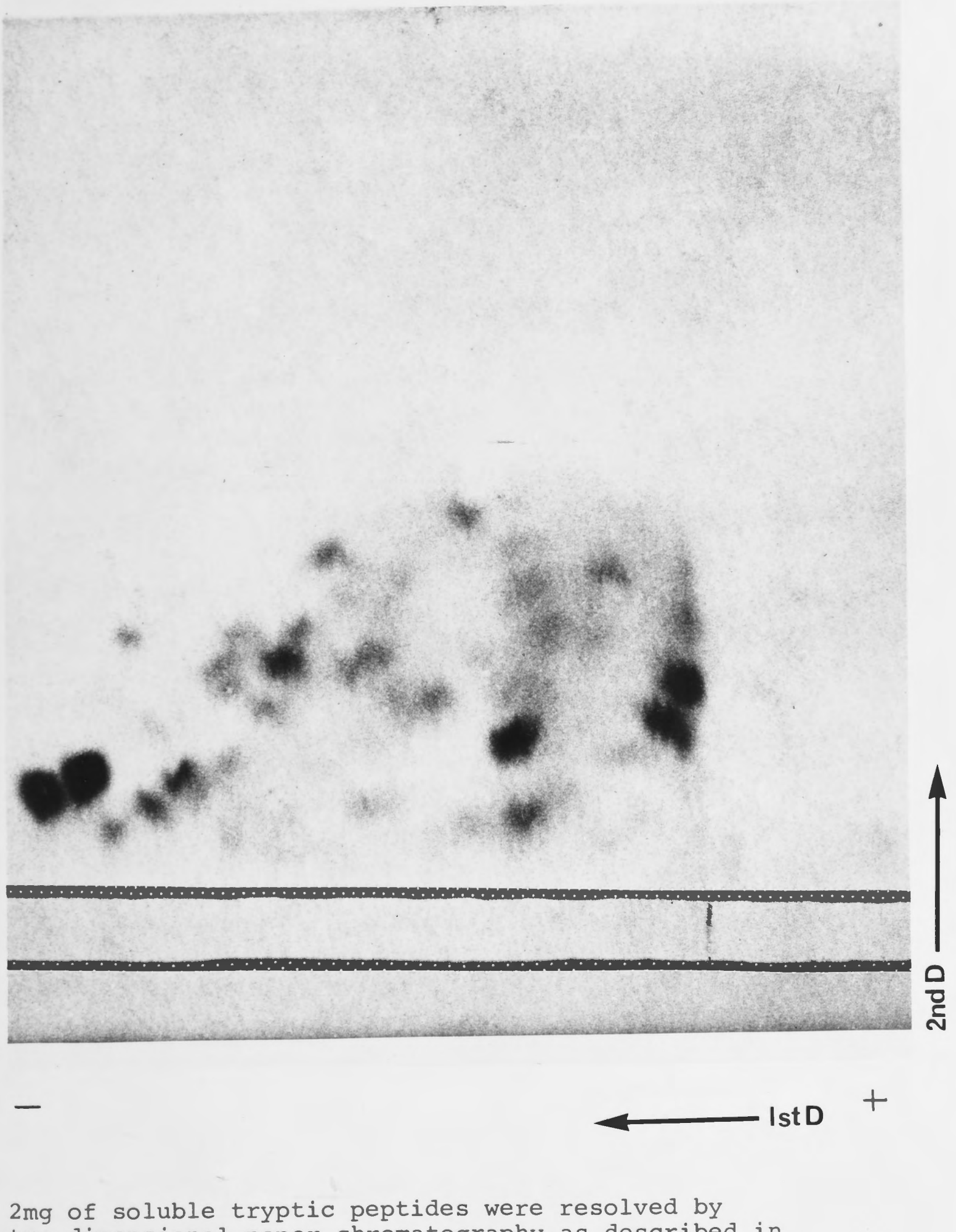
TABLE 5-3. Amino Acid Analysis of Tryptic Core Peptides

	pH4 core		pH8 core	
	nmoles	ng	nmoles	ng
Lys	10.463	1341	31.377	4023
His	8.622	1183	15.287	2097
Arg	9.430	1473	24.386	3809
Asx[a]	30.320	3490	45.620	5251
Thr	8.339	843	27.738	2804
Ser	15.215	1325	37.799	3292
Glx	21.939	2832	44.517	5747
Pro	5.866	570	17.884	1737
Gly	25.528	1458	65.640	3748
Ala	20.003	1422	60.935	4332
Cya	2.380	246	4.600	475
Val	17.610	1745	48.097	4766
Met[a]	-	-	28.763	3774
Ile	16.222	1836	42.741	4838
Leu	28.698	3249	78.922	8934
Tyr	6.976	1138	21.158	3453
Phe	12.855	1892	31.218	4595
		26043		67675

6.6mg (or ~150nmoles) of NADH:ubiquinone oxidoreductase was digested with trypsin as described in the text. The pH4 and pH8 core peptides were collected by centrifugation, hydrolyzed in 6N HCl at 110° for 22h, then 1/10 of this material analyzed. From the above analysis, the yield pH4 core = 0.26mg; pH8 core = 0.68mg. From the arginine and lysine content there is a possibility of 2 and 5 tryptic peptides (at the 100nmole level) in the pH4 and pH8 core respectively.

[a]Asx + Met : aspartic acid and methionine sulphone were insufficiently resolved to permit separate quantification.

FIGURE 5-2. Two Dimensional Paper Chromatography of NADH:ubiquinone Oxidoreductase Soluble Tryptic Peptides



2mg of soluble tryptic peptides were resolved by two dimensional paper chromatography as described in the text. The above peptide map was stained with ninhydrin.

25 residues per polypeptide respectively) a total of 48 tryptic peptides would be predicted. Consideration of the amino acid sequence however (Figure 5-3) indicates that, due to the presence of various arg-arg, lys-pro *etc*. sequences, only 39 tryptic peptides plus free arginine and lysine would be produced. This is in agreement with the number of tryptic peptides visible, which in turn suggests that the enzyme is not composed of two dissimilar polypeptides of identical molecular weight, since if this were the case, double the number of tryptic peptides would be expected. (Note: this does not preclude there being two non-identical though related polypeptides of approximately the same molecular weight).

Nine tyrosine containing peptides were detected when the aforementioned peptide map was specifically stained, following ninhydrin staining, with α-nitroso-β-naphthol (Easley, 1965). This is exactly the number one would predict from the expected tryptic peptides produced as a consequence of the amino acid sequence.

A separate tryptic map was produced with 0.5mg of the soluble tryptic peptides and stained consecutively with Fluram, and then specifically for histidine (Easley, 1965) and arginine (Yamada & Itano, 1966) containing peptides. A total of 8 histidine and 13 arginine-containing peptides were visualized, which compares with 13 and 19 respectively, expected from a consideration of the amino acid sequence. It is possible that a maximum of 2 histidine and 3 arginine-containing peptides are present in the core peptides (Table 5-3).

FIGURE 5-3. Amino Acid Sequence of NADH:ubiquinone Oxidoreductase Showing Location of Isolated Tryptic and CNBr Peptides

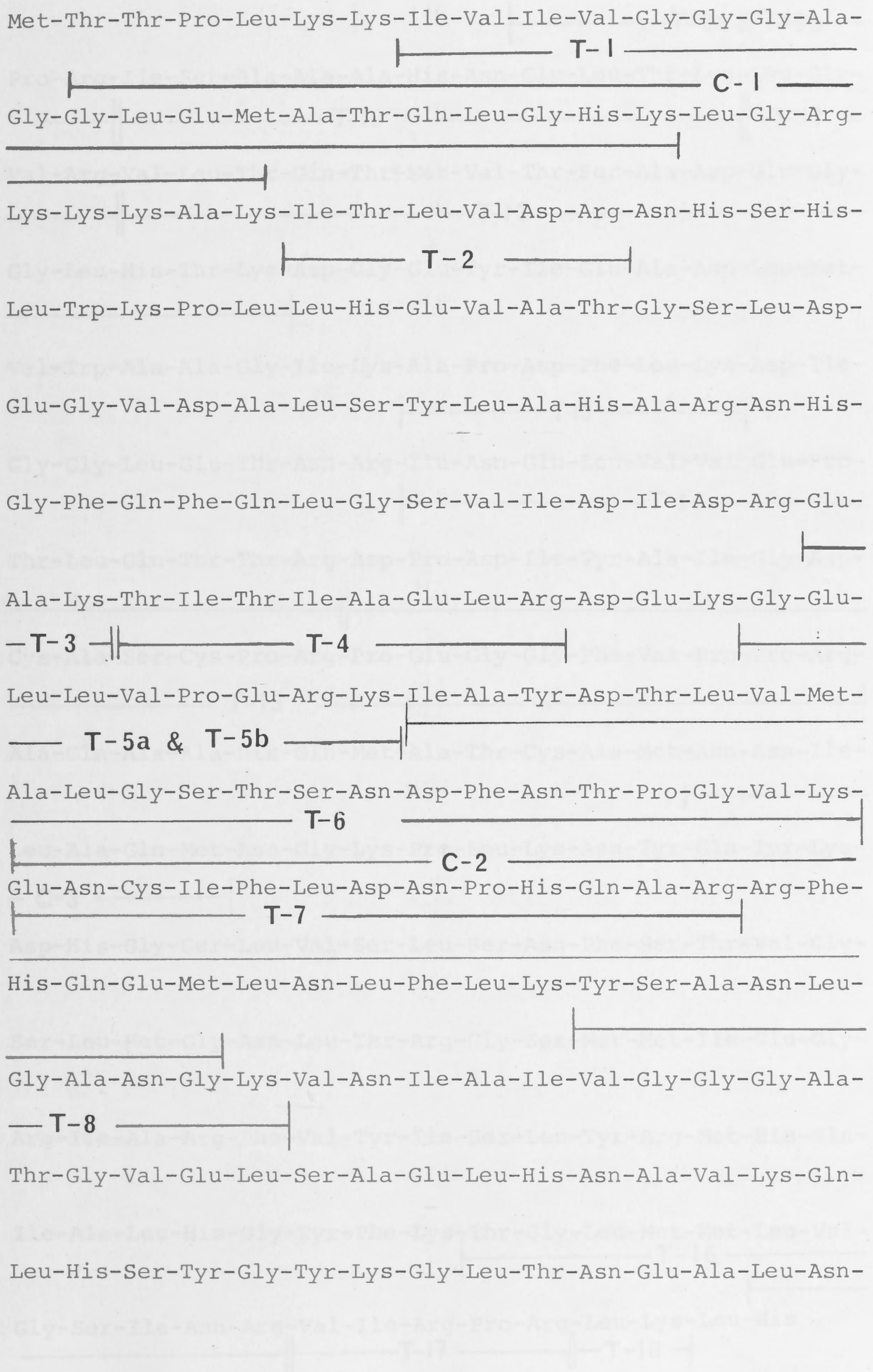
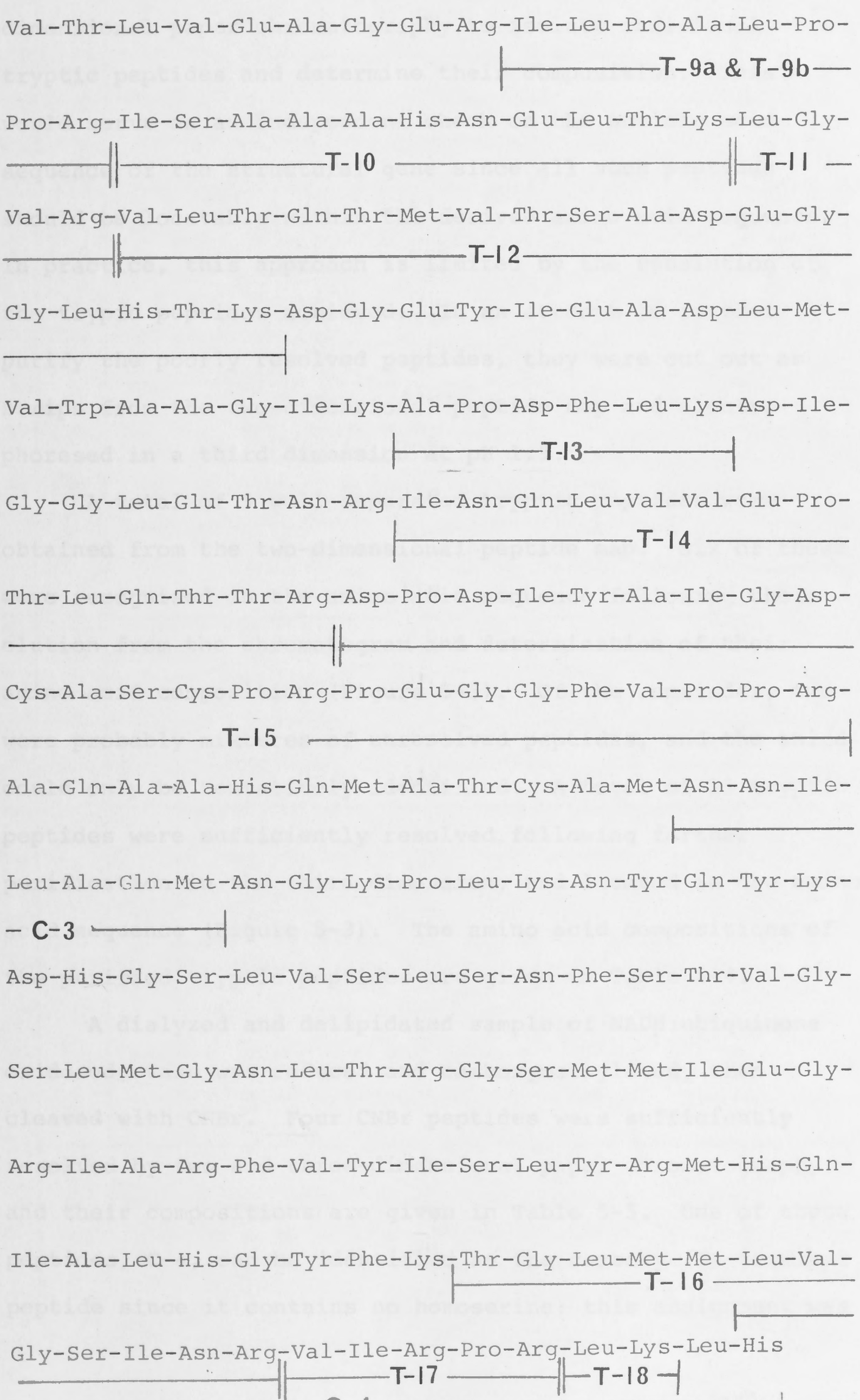


FIGURE 5-3 Continued.



Amino Acid Analysis of CNBr and Tryptic Peptides

It was decided to use the techniques of two and three dimensional paper chromatography to isolate individual tryptic peptides and determine their composition. This would serve as an independent check on the nucleic acid sequence of the structural gene since all such peptides should be locatable within the derived amino acid sequence. In practice, this approach is limited by the resolution of the trypic peptides achieved. In an attempt to further purify the poorly resolved peptides, they were cut out as strips from the two-dimensional peptide map and electrophoresed in a third dimension at pH 1.9.

A total of 9 well-separated tryptic peptides were obtained from the two-dimensional peptide map. Six of these were recognised in the amino acid sequence following their elution from the chromatogram and determination of their amino acid composition (Figure 5-3). Of the remainder, two were probably mixtures of unresolved peptides, and the third could only be tentatively identified. A total of 15 tryptic peptides were sufficiently resolved following further purification in the third dimension, and located in the amino acid sequence (Figure 5-3). The amino acid compositions of the purified tryptic peptides are given in Table 5-4.

A dialyzed and delipidated sample of NADH:ubiquinone oxidoreductase was reduced and carboxymethylated, then cleaved with CNBr. Four CNBr peptides were sufficiently resolved by two and three-dimensional paper chromatography, and their compositions are given in Table 5-5. One of these peptides, C-4, can be identified as the carboxy (C)-terminal peptide since it contains no homoserine; this assignment was

TABLE 5-4. Amino Acid Composition of Tryptic Peptides

Amino acid	Peptide						
	T-1	T-2	T-3	T-4	T-5(a)	T-5(b)	T-6
Cys[a]							
Asx	(0.56)	1.01(1)					4.94[c]
Thr	1.06(1)	0.97(1)	0.78(1)	1.77(2)			1.64(3)
Ser	1.03(1)						1.84(2)
Glx	2.40(2)			1.37(1)	1.57(2)	2.84(2)	1.19(1)
Pro					0.72(1)	0.80(1)	1.23(1)
Gly	5.49(6)				0.69(1)	1.15(1)	2.70(2)
Ala	1.95(2)		0.95(1)	1.12(1)			2.52(2)
Val	1.35(1)	1.01(1)			0.82(1)	0.84(1)	2.14(2)
Met[b]	1.12(1)						-[c]
Ile	1.10(2)	0.81(1)		1.84(2)			0.87(1)
Leu	2.19(1)	1.15(1)		1.25(1)	1.72(2)	1.82(2)	2.76(2)
Tyr							
Phe							0.97(1)
His	1.22(1)						
Lys	1.00(1)		1.00(1)		1.00(1)	1.00(1)	1.00(1)
Arg		1.00(1)		1.00(1)	0.73(1)	0.88(1)	(0.29)
nmoles	3.230	7.588	10.230	2.035	2.172	2.219	3.427

Continued.

TABLE 5-4 Continued.

Amino acid	Peptide						
	T-7	T-8	T-9(a)	T-9(b)	T-10	T-11	T-12
Cys[a]	1.00(1)						
Asx	3.12(3)	2.23(2)			1.07(1)		1.06(1)
Thr					0.90(1)		2.55(4)
Ser		1.22(1)			1.21(1)		0.97(1)
Glx	2.12(2)	(0.57)	(0.33)		1.66(1)		2.04(2)
Pro	0.99(1)		2.38(3)	2.70(3)			
Gly	(0.35)	2.36(2)	(0.33)	(0.35)	(0.67)	1.13(1)	1.95(2)
Ala	1.27(2)	2.24(2)	1.02(1)	1.12(1)	2.47(3)		1.20(1)
Val						0.87(1)	1.41(2)
Met[b]							0.81(1)
Ile	0.90(1)		0.90(1)	0.88(1)	(0.50)		
Leu	1.12(1)	1.18(1)	2.84(2)	2.24(2)	1.26(1)	1.04(1)	1.73(2)
Tyr		0.53(1)					
Phe	0.89(1)						
His	0.91(1)				0.88(1)		0.50(1)
Lys		1.00(1)			1.00(1)	(0.28)	1.00(1)
Arg	1.00(1)		1.00(1)	1.00(1)		1.00(1)	
nmoles	6.561	2.831	6.018	5.537	1.421	8.878	6.999

Continued.

TABLE 5-4 Continued.

Amino acid	Peptide						
	T-13	T-14	T-15	T-16	T-17	T-18	T-19
Cys[a]			2.15(2)				
Asx	0.94(1)	1.36(1)	3.24(3)	1.15(1)			
Thr		3.07(3)		0.92(1)			
Ser			1.18(1)	1.30(1)			
Glx		3.22(3)	1.51(1)	(0.69)			
Pro	1.01(1)	1.05(1)	4.81(5)		0.95(1)		
Gly		(0.40)	3.23(3)	2.92(2)			
Ala	0.87(1)	(0.25)	2.18(2)	(0.58)			
Val		1.34(2)	1.01(1)	1.23(1)	0.44(1)		
Met[b]				2.28(2)			
Ile		0.84(1)	1.83(2)	1.07(1)	0.49(1)		
Leu	1.16(1)	2.26(1)		2.39(2)		0.73(1)	
Tyr			0.51(1)				
Phe	0.93(1)		0.94(1)				
His							
Lys	1.00(1)					1.00(1)	1.00
Arg		1.00(1)	2.00(2)	1.00(1)	2.00(2)		
nmoles	8.617	7.736	11.062	2.977	10.524	10.343	30.604

Continued.

TABLE 5-4 Continued.

Tryptic peptides were purified by two and three dimensional paper chromatography (see text for details). Values for each amino acid are normalized to either the lysine or arginine content: residues present at a level less than 0.25moles/mole peptide are not listed. Levels of the peptides (based on either the lysine or arginine content) are given in the last row. Major contaminants are given in parentheses.

[a]Estimated as cysteic acid.

[b]Estimated as methionine

[c]Asx + Met: aspartic acid and methionine sulphone insufficiently resolved for separate quantification.

TABLE 5-5. Amino Acid Composition of Purified CNBr Peptides

Amino Acid	Peptide			
	C-1	C-2	C-3	C-4
Lys	16.3(2)	25.0(1)		8.3(1)
His		12.5(1)		8.7(1)
Arg		36.2(2)		25.9(3)
Asx		50.1(4)	12.5(1)	10.5(1)
Thr	18.7(2)	54.6(2)		
Ser		33.4(2)		10.0(1)
Glx	12.4(1)	84.9(4)	11.8(1)	
Pro	8.5(1)	64.6(3)		8.1(1)
Gly	49.5(5)	70.6(3)	7.9(1)	12.2(1)
Ala	10.8(1)	84.7(4)	9.8(1)	
Cys[a]		8.0(1)		
Val	16.9(2)	34.1(2)		14.9(2)
Met				
Ile	15.0(2)	42.3(2)	9.1(1)	14.2(2)
Leu	21.3(2)	48.3(2)	11.0(1)	26.2(3)
Tyr		13.5(1)		
Phe		19.6(1)		
Trp				
Hse[b]	5.6(1)	11.0(1)	6.9(1)	

CNBr peptides were analyzed following hydrolysis in 6N HCl at 110° for 22h. The amount (nmoles) of each amino acid is listed, with the probable number per peptide in parentheses. Residues present at less than the 3nmole level are not listed. For further details see text.

[a]Estimated as carboxymethyl cysteic acid.

[b]Homoserine plus homoserine lactone.

subsequently borne out by the sequence data (Figure 5-3). All four CNBr peptides were located in the amino acid sequence (Figure 5-3), and one of these, C-1, was positioned at the amino (N) terminus.

The fact that both the N and C-terminal peptides have been isolated is good evidence that significant proteolysis has not occurred during enzyme purification, which supports the evidence obtained by SDS polyacrylamide gel electrophoresis (see Chapter 4).

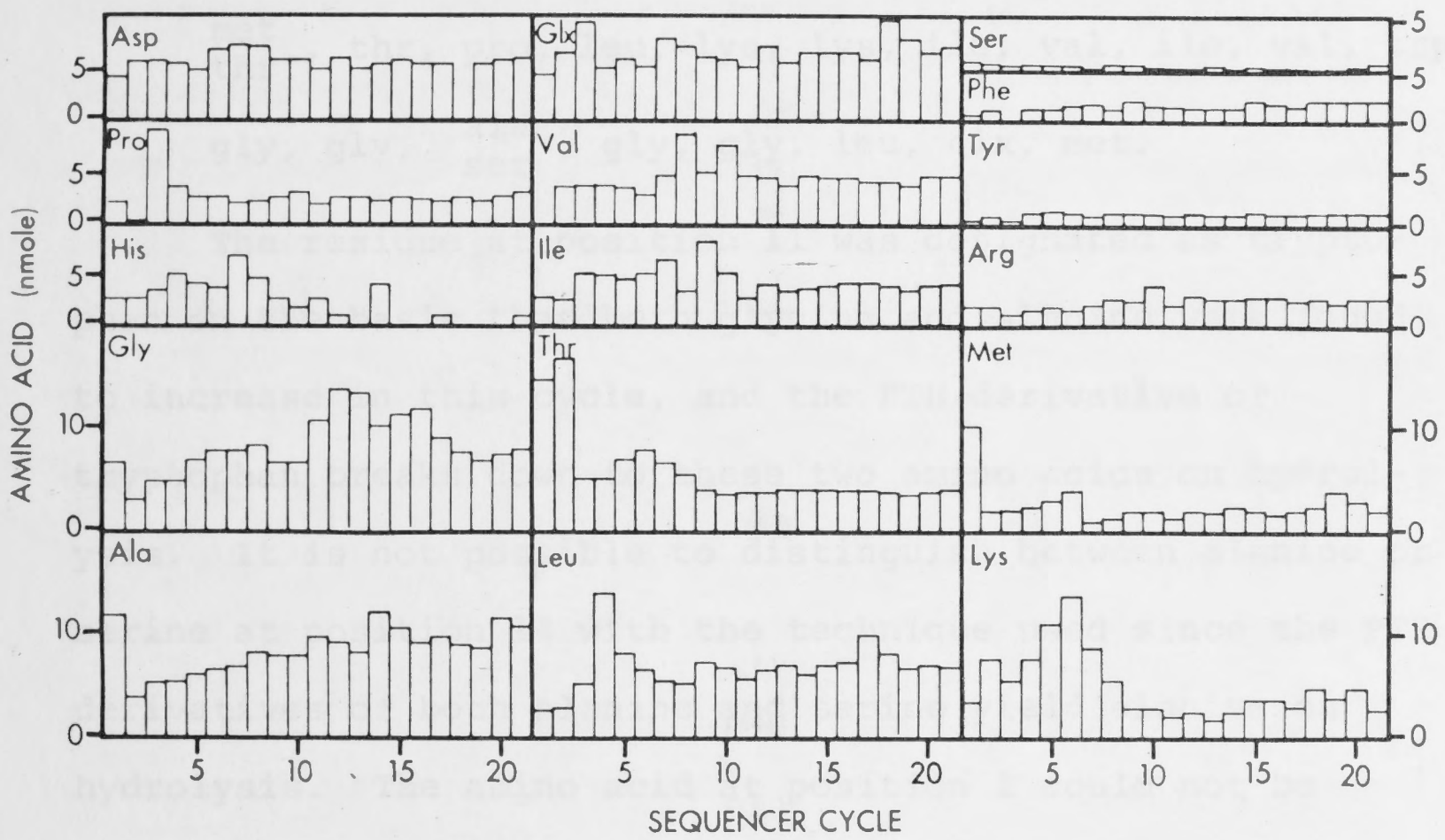
The tryptic and CNBr peptides together constitute 49.8% of the polypeptide chain, sufficient to verify that there are no major flaws in the nucleic acid sequence. It is interesting that most of the peptides isolated and purified seem to fall within four or five major regions in the primary sequence.

N-terminal Amino Acid Sequence of NADH:ubiquinone Oxidoreductase

The primary structure of the N-terminus was obtained by direct sequence determination on reduced and carboxymethylated NADH:ubiquinone oxidoreductase in a Beckman 890C sequencer. The residues released after each cycle were determined by amino acid analysis after hydrolysis of the corresponding PTH derivatives.

The results of the first 21 cycles of one such experiment are shown in Figure 5-4. Interpretation of the data was rendered difficult by the fact that the background concentration of amino acids was high and by the fact that the yield of the true sequence was low. One reason for this appears to be the incomplete solubility of the carboxymethylated enzyme in quadrol buffer, but the possibility was also

FIGURE 5-4. Amino Acid Sequencer Data for the First 20 Residues of NADH:ubiquinone Oxidoreductase



Amino acid analysis of the first 21 cycles of a single sequencer experiment on NADH:ubiquinone oxidoreductase. Deduced sequence: ·thr.pro.leu.lys.lys.ile.val.ile.val.trp.gly.gly.$\begin{matrix}\text{ala}\\\text{ser}\end{matrix}$.gly.gly.leu.glx.met... Threonine was determined as γ-amino isobutyric acid and the isoleucine values were obtained from the sum of the isoleucine and *allo*-isoleucine peaks. See text for details.

considered that a considerable proportion of the enzyme in the preparation has a blocked N-terminus (see below). The high background may result from a partial degradation of the polypeptide during delipidation.

All in all, the sequence of the first 19 amino acids was determined in 2 separate experiments and that of the first 8 amino acids in 3 separate experiments. From these preliminary experiments the following sequence was deduced:

$$\overset{\text{met}}{\text{thr}}, \text{ thr, pro, leu, lys, lys, ile, val, ile, val, trp,}$$

$$\text{gly, gly, } \overset{\text{ala}}{\text{ser}}, \text{ gly, gly, leu, glx, met.}$$

The residue at position 11 was designated as tryptophan on the basis that both glycine and alanine were found to increase in this cycle, and the PTH derivative of tryptophan breaks down to these two amino acids on hydrolysis. It is not possible to distinguish between alanine or serine at position 14 with the technique used since the PTH derivatives of both alanine and serine yield alanine on hydrolysis. The amino acid at position 1 could not be unequivocally assigned since glycine, alanine, threonine (as γ-amino isobutyric acid) and methionine were all detected in this cycle. Free glycine and alanine (from serine) are likely contaminants in the preparation, so their presence is not unexpected. Threonine is less likely to be a contaminant, although like serine it can arise by partial acid cleavage involving N $\longrightarrow$ O acyl migration. A tentative assignment of methionine at position 1 was made.

The prediction of the N-terminal sequence in these early experiments was found to be remarkably accurate, considering the quality of the data, when the corresponding

nucleic acid sequence data was finally obtained (*c.f.* above and Figure 5-3). The nucleic acid sequence corrected the interpretation of the residue at position 11, and decided between the alternatives at position 14. By far the most important finding predicted by the nucleic acid sequence, is that the first residue in the protein is threonine, and not methionine as assigned above*. Presumably, therefore, the N-terminal formyl-methionine is wholly or partially processed (*i.e.* cleaved) in the preparation†. A related explanation considered was that the enzyme has a short signal peptide (for a review see Davis & Tai, 1980) which is not cleaved in a proportion of enzyme molecules. Such signal peptides, or leader sequences, are usually 16 to 20 amino acids long and serve as a mechanism for the trans-membrane insertion of both excreted proteins and membrane-bound proteins (ibid.). If such a signal peptide were still present on a proportion of enzyme molecules, then this could help explain how the pure preparation can reconstitute NADH oxidase in mutant membrane particles (see Chapter 4). This explanation was not considered the most likely since the processed and unprocessed enzyme, unlike the case where processing involves the cleavage of only an N-terminal formyl-methionine, should be resolved on SDS polyacrylamide

---

*This, as well as the other corrections, has been confirmed following the sequencing of the first 36 residues of NADH: ubiquinone oxidoreductase (D.C. Shaw & H.D. Campbell, unpublished results). The quality of the data was considerably improved when the enzyme was delipidated by chromatography on a Sephacryl S300 column in the presence of 1% (w/v) SDS.

†Any enzyme molecules which have formyl-methionine as their N-terminus would not react with phenylisothiocyanate, and the presence of such unprocessed enzyme would manifest itself in a low sequence yield, as was observed.

gel electrophoresis.

The codon immediately preceding the first threonine codon in the nucleic acid sequence, is not the initiation codon, AUG, but is instead a leucine codon, UUG (Figure 5-5). The designation of this UUG as the initiation codon in the *ndh* gene is strengthened by the presence of a very strong ribosome binding site ("Shine-Dalgarno sequence") starting 13 bases towards the 5' end of the UUG and which has 9 bases complementary to the 3' end of the 16S rRNA of *E. coli*.

There is no other candidate for an initiation codon in the sequence*.

The question which must be asked is whether the UUG specifies a formyl-methionine as the first amino acid, and whether translation is initiated by the normal $tRNA^{f\text{-}met}$. The existence of both methionine and threonine in the first sequencer cycle can be interpreted as there being enzyme molecules in the preparation in which either the formyl group or the formyl-methionyl residue has been cleaved, yielding sequences beginning with methionine and threonine respectively. A small yield of the sequence beginning with methionine would not be detected, except at cycle 1, against a larger yield of the sequence beginning with the first threonine, since the former is only one amino acid out of phase with the latter and would be obscured by the 'ghosting' normally observed after each cycle. It is likely that if partial processing of the enzyme is the case, this is a

---

*There is an AUG 51 bases 'upstream' from the UUG codon, which would fit the model that NADH:ubiquinone oxido-reductase has a signal polypeptide, but it is separated from the coding sequence by termination codons in all three reading frames.

FIGURE 5-5. Nucleic Acid Sequence at the 5' of the *Ndh* Gene

.... T A A G G G T C A C G T T G A C T A C G C C A T T G
(met) thr thr pro leu

A A A A A G A T T G T G A T T G T C G G C G G C G G T
lys lys ile val ile val gly gly gly

G C T G G T G G G C T G G A A A T G G C A A C A .....
ala gly gly leu glu met ala thr

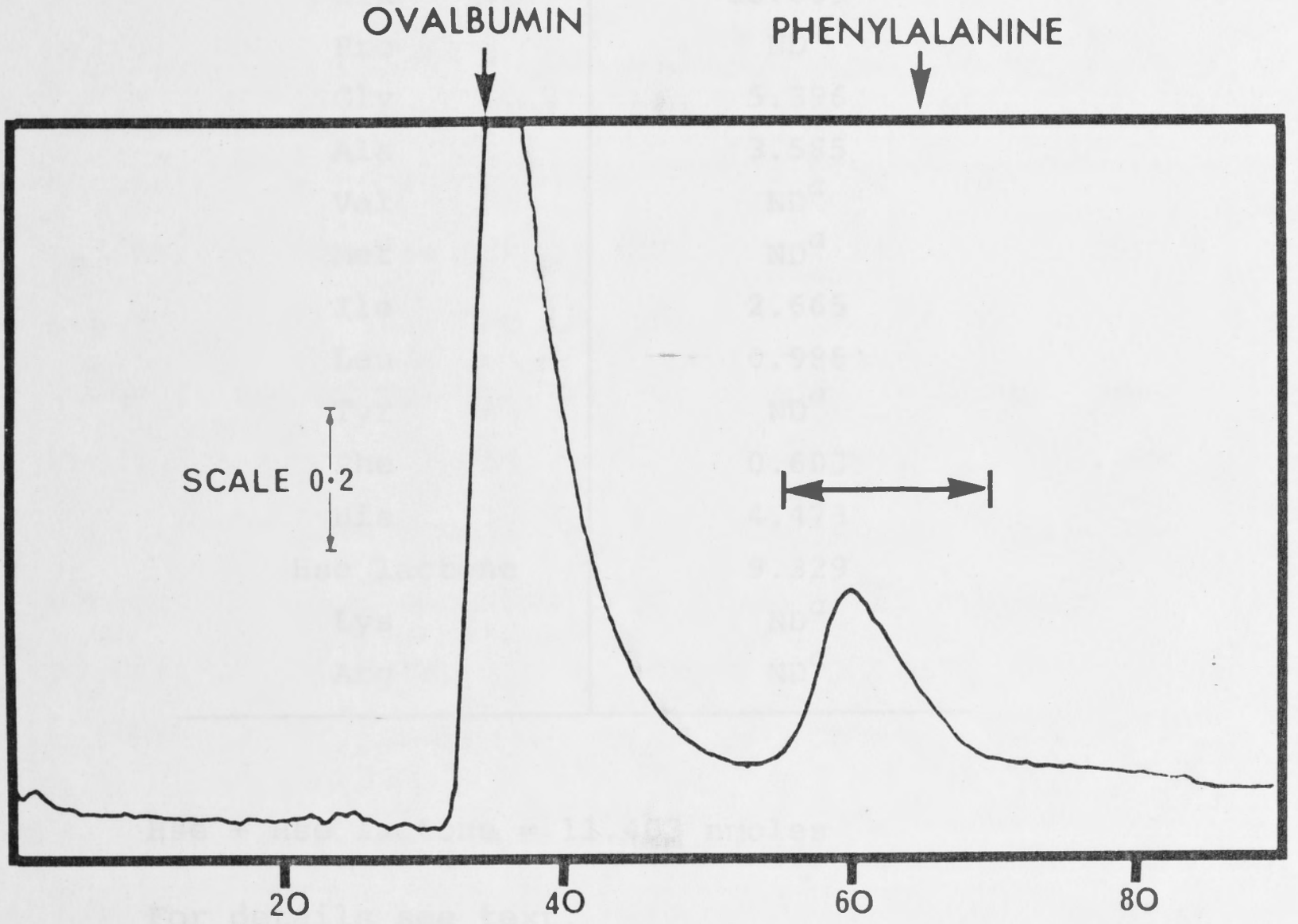
The sequence homologous to the 16S rRNA of E. coli, the possible ribosome binding site, is underlined.

result of the very high amplification of the enzyme levels achieved *in vivo* exceeding the capacity of the processing machinery in the cell. Such a phenomenon has recently been reported for the tryptophan synthetase *a*-subunit purified from genetically amplified strains of *E. coli* (Sugino *et al.*, 1980).

Demonstration of the Amino-Terminal Formyl-Methionine

Experiments were undertaken, aimed at identifying the formyl-methionine at the N-terminus of the unprocessed enzyme. Approximately 2mg of carboxymethylated NADH: ubiquinone oxidoreductase was cleaved with CNBr, and the reaction products chromatographed on a column of Sephadex G25 (Figure 5-6). The low molecular weight material was pooled and chromatographed on a column of Dowex-50x2 (H^+ form), eluted with H_2O, then analyzed. The rationale behind this procedure is that CNBr should cleave the enzyme, producing formyl-homoserine, derived from the N-terminal formyl-methionyl residue, and various CNBr peptides. After collection of the low molecular weight fraction, the formyl-homoserine is further purified by making use of its lack of a free amino group, This step is necessary because, as was shown later, the protein may contain met.met sequences which produce free homoserine on cleavage with CNBr: such material is retained on the Dowex column. The presence of formyl-homoserine in the H_2O eluate was established by amino acid analysis following acid hydrolysis to remove the formyl group. The results of the analysis are shown in Table 5-6. A total of ~11.5nmoles of homoserine plus homoserine lactone was recovered from ~45nmoles of carboxymethylated enzyme (these results should only be seen as an approximation, see

FIGURE 5-6. Sephadex G25 Chromatography of NADH: ubiquinone Oxidoreductase CNBr Peptides



A $_{280}$ trace of CNBr peptides separated on Sephadex G25 was obtained with an Isco Model UA-5 absorbance monitor. The column was pre-calibrated with ovalbumin and phenylalanine. Fractions 56 to 70 were pooled (vol. 7.5mL). For further details see Experimental section.

TABLE 5-6. Amino Acid Analysis of Dowex 50x2 H_2O Eluate

Amino Acid	nmoles
Asx	1.277
Thr	0.793
Ser	2.350
Hse	2.074
Glx	38.809
Pro	ND[a]
Gly	5.396
Ala	3.585
Val	ND[a]
Met	ND[a]
Ile	2.665
Leu	0.986
Tyr	ND[a]
Phe	0.603
His	4.473
Hse lactone	9.329
Lys	ND[a]
Arg	ND[a]

Hse + Hse lactone = 11.403 nmoles

For details see text.

[a]non detected.

Experimental section). This indicates that about 25% of the enzyme in the sample hydrolyzed may have a blocked N-terminus.

The amino acid analysis also showed a high yield of glutamic acid and lesser amounts of glycine, alanine and histidine, but no methionine. The presence of glutamic acid is expected since glutamine can cyclize to form pyrrolidone carboxylic acid, which has no free amino group.

It is stressed that this is only an indirect demonstration of formyl-methionine in the preparation and that, even when taken into consideration with the sequence data, this has not proven that the UUG initiation codon is specific for formyl-methionine (although this remains the most likely model at present). To this end experiments have been in progress to try to isolate the unprocessed N-terminal tryptic peptide and sequence it by mass-spectrometry and to identify the N-terminal di- or tri-peptide by GC-mass-spectrometry.

SUMMARY

The amino acid analysis of NADH:ubiquinone oxido-reductase purified from *E. coli* membrane particles is in agreement with that predicted from the amino acid sequence. Together with the results obtained by peptide mapping, it has been confirmed that the enzyme is composed of a single type of polypeptide chain. The tight binding of the enzyme to the membrane cannot be explained simply on the basis of

the hydrophobicity of the overall protein.

Amino acid composition data have been obtained for tryptic peptides and CNBr peptides covering 49.8% of the primary sequence, serving as a check on the nucleic acid sequence. As well, CNBr peptides have been obtained covering both the amino and carboxyl-termini, confirming (when taken into account with the results of SDS polyacrylamide gel electrophresis) the integrity of the polypeptide chain, as isolated.

The sequence of the first 21 residues of the processed enzyme has been determined. Unequivocal identification of formyl-methionine as the amino terminus of the unprocessed enzyme has not been achieved, and so direct assignment of the UUG leucine codon as the initiation codon for the *ndh* gene cannot be made from the protein chemical data.

CHAPTER 6.

General Discussion

In the experiments described in Chapter 2, crude *E. coli* membrane particles were solubilized with 3% potassum cholate and 1M KCl at 0 to 4°. It was hoped that by using a gentle solubilization procedure, the NADH: ubiquinone oxidoreductase segment of the respiratory chain could be solubilized intact. Good recovery of NADH: ubiquinone oxidoreductase activity was obtained, and when solubilized material from the wild-type strain IY13 was chromatographed on hydroxylapatite, a single major peak of NADH-dependent ubiquinone reductase activity was resolved. This activity is derived from the respiratory NADH dehydrogenase complex since it was shown to be absent in *ndh* mutant strains, and therefore the corresponding enzyme preparation contains the respiratory NADH dehydrogenase.

The single-column purification procedure results in an increase of 30 to 40-fold in specific activity relative to wild-type membrane particles. This is mainly due to unusually tight binding of the enzyme to hydroxylapatite under the conditions used. Further purification of the enzyme proved to be difficult however, and large losses of activity were incurred with all of the approaches subsequently tried. Attempts were therefore made to amplify the levels of the enzyme *in vivo*, to facilitate purification, by using various genetic approaches.

During attempts to clone the *ndh* gene onto multicopy cloning vectors, two hybrid plasmids were constructed which complement the *ndh* mutant phenotype (Young *et al.*, 1978). As shown in Chapter 3, strains carrying one of these hybrid plasmids, pIY1, possess high levels of NADH:ubiquinone

oxidoreductase in their cell membranes. The levels of the cyanide-sensitive, membrane-bound NADH oxidase activity are also increased, which indicates that the enzyme, whose levels have been amplified in this manner, can interact with the respiratory chain. The amplified enzyme elutes in an identical position, during hydroxylapatite column chromatography, to the NADH:ubiquinone oxidoreductase absent in IY12, and by this criterion, is derived from the respiratory NADH dehydrogenase complex. Analysis of the enzyme preparation from such genetically-amplified strains shows a single polypeptide, subsequently confirmed by the protein chemical studies in Chapter 5, of molecular weight ~45,000. This polypeptide can therefore be unequivocally identified as the respiratory NADH dehydrogenase.

The cloned DNA fragment in pIY1 contains the structural gene coding for the respiratory NADH dehydrogenase. This was initially demonstrated when it was shown that both the hybrid plasmid and the cloned DNA insert itself can direct the synthesis of the 45,000 molecular weight polypeptide chain in an *E. coli* cell-free transcription/translation system (M.I. Poulis, unpublished results). These studies also indicated that the promoter region for the *ndh* gene has been cloned intact. A direct proof that the *ndh* gene has been cloned comes ultimately from a comparison of the nucleic acid sequence of the insert with the amino acid composition and protein sequence data on the purified NADH dehydrogenase (see Chapter 5).

The NADH dehydrogenase preparation has a very high activity with ubiquinone as electron acceptor, a (relatively) low activity with potassium ferricyanide, and

is specific for NADH as electron donor. The data presented in Chapter 4 indicates that the enzyme contains a single molecule of non-covalently-bound FAD per polypeptide chain but no Fe or acid-labile S. Other metals assayed for but not detected include W, Cu and Mo. This suggests that there are no iron-sulphur clusters or other metal complexes which could act as electron acceptors and participate in catalysis. The only metals not assayed, but which have been found in other respiratory enzymes, are Se (in enzymes such as formate dehydrogenase, Enoch & Lester, 1975) and Zn (found in various D-lactate dehydrogenase flavoproteins, Olson & Massey, 1979; Gregolin & Singer, 1963). There is also no evidence for iron-sulphur or haem prosthetic groups from the visible spectrum of the pure enzyme. The question which must be asked is whether the purified enzyme contains all of the components (polypeptides and prosthetic groups) involved in electron transport between NADH and the endogenous ubiquinone pool, or whether the very high ubiquinone reductase activity of the preparation is artifactual.

The amplification in the levels of respiratory NADH dehydrogenase causes an increase in the membrane-bound NADH: ubiquinone oxidoreductase activity which is consistent with that expected from the increase in the *ndh* gene copy-number. This indicates that the ubiquinone reductase activity of the NADH dehydrogenase is not generated by solubilization or subsequent column chromatography. The demonstration that the specific activity of the pure enzyme from chloramphenicol-amplified IY35 or IY85 strains is similar to that of the IY13 enzyme preparation (assuming one active

site per 45,000 molecular weight subunit in the latter) is good evidence that an alteration in the catalytic properties of the enzyme has not occurred as a result of amplification.

All of the *ndh* mutants isolated so far are defective in the respiratory NADH dehydrogenase flavoprotein. The failure to detect any other class of mutant defective in the respiratory NADH dehydrogenase complex is consistent with the assumption that there are no other component enzymes located between the primary NADH dehydrogenase and the ubiquinone pool, although since only a dozen *ndh* mutants have been isolated so far, such evidence may not be considered strong, and of course cannot be conclusive.

It is reasonable to postulate that the respiratory NADH:ubiquinone oxidoreductase has been purified intact. This rests on the assumption that the measurement of exogenous ubiquinone reduction by membrane particles assays this segment of the respiratory chain. This assumption has been shown to be reasonable in the case of mitochondrial membrane particles (Hatefi *et al.*, 1962; Ragan & Hinkle, 1975; Schatz & Racker, 1966), and energy conservation at site 1, linked to the reduction of exogenous ubiquinone-1, has been demonstrated in mitochondrial particles (Schatz & Racker, 1966) and vesicles reconstituted from Complex 1 and phospholipid (Ragan & Hinkle, 1973).

There has been a great deal of controversy in the literature concerning the attempts to purify NADH: ubiquinone oxidoreductase from mitochondria, and in particular whether the various soluble low molecular weight (type 2) NADH dehydrogenases, which are able to reduce

ubiquinone analogues (Pharo *et al.*, 1966; Raw *et al.*, 1961; Kumar *et al.*, 1968; Hatefi & Stempel, 1969) are intact preparations of this segment of the respiratory chain. Singer and co-workers strongly hold the view that such low molecular weight dehydrogenases are fragments of the enzyme complex which reduces ubiquinone *in vivo*, and that the ubiquinone reductase activity they possess is an artifact of solubilization (for a review of their arguments see Singer & Gutman, 1971). The major experimental evidence for this assertion comes from the demonstration that these soluble ubiquinone reductases can be isolated by treatment of high molecular weight (type 1) NADH dehydrogenase, which itself has no ubiquinone reductase activity, under the same conditions as permits their solubilization from mitochondrial particles (Cremona *et al.*, 1963; Machinist & Singer, 1965; Kaniuga, 1963; Cremona & Kearney, 1963).

There are major differences in the nature of the preparations of *E. coli* NADH:ubiquinone oxidoreductase reported in this work, on the one hand, and the soluble type 2 NADH dehydrogenase from mitochondria, on the other. The former contains approximately 70% lipid by weight whereas the latter, isolated by harsher procedures, are devoid of lipid. The physiological reduction of ubiquinone in mitochondrial preparations has been shown to require phospholipid, and this may reflect a hydrophobic interaction of the substrate with the enzyme (Ragan & Racker, 1973), therefore this reaction may not be amenable to study in a soluble, lipid-free preparation (Singer & Gutman, 1971). This suggests that the strategy of trying

to purify this segment of the respiratory chain in a water soluble form has been misguided. The strategy used in the present work has been to isolate the enzyme as a lipo-protein complex, similar in nature to Complex 1, by using gentle solubilization procedures. The difficulty in this approach, as illustrated by the work of Hatefi and co-workers, is that large aggregates, which are difficult to further resolve, can be solubilized, since the relatively strong protein-lipid interactions are not totally disrupted. The unique use of genetic engineering techniques in the present work has enabled the NADH:ubiquinone oxido-reductase to be purified to homogeneity without delipidation of the preparation at any stage, thereby protecting the labile ubiquinone reductase activity.

Irreversible denaturation of the mitochondrial enzyme following delipidation under aerobic conditions has been demonstrated (Ohnishi *et al.*, 1974): it appears that this is due to oxidation of a sensitive iron-sulphur centre, N-2 (Orme-Johnson *et al.*, 1971), which has been postulated to be the immediate electron donor to ubiquinone in mitochondria. It has recently been possible to study the ubiquinone reductase reaction in delipidated preparations of mitochondrial succinate:ubiquinone oxidoreductase by incorporating this enzyme into triton micelles (Weiss & Kolb, 1979; Weiss & Wingfield, 1979). The NADH:ubiquinone oxidoreductase preparation described in this work is amenable to manipulation, and it should be possible to study its catalytic properties in a more defined system by incorporating the enzyme into either detergent micelles or artificial phospholipid vesicles.

It appears that the *E. coli* NADH:ubiquinone oxidoreductase is not an iron-sulphur flavoprotein since, as was shown in Chapter 4, no Fe or acid-labile S was detected in preparations possessing full ubiquinone reductase activity, and although the possibility cannot be ruled out that Fe or acid-labile S has been lost during solubilization and hydroxylapatite column chromatography of the enzyme, it is reasonable to assume that Fe is probably not involved in electron transport between NADH and ubiquinone in *E. coli*.

The structure of the respiratory NADH:ubiquinone oxidoreductase bears a strong resemblance to that of the microsomal NADH:cytochrome b_5 reductase (Williams, 1976). Both enzymes have a single polypeptide chain of approximately equal molecular weight, a single non-covalently-bound FAD per subunit, and no Fe or acid-labile S. Both are membrane-bound, reduce membrane bound substrates, and are NADH specific. A mechanism has been proposed for NADH:cytochrome b_5 reductase based on sequential $1e^-$ transfer to cytochrome b_5 involving an intermediate flavosemiquinone (Strittmatter, 1965). It may be possible that an analogous mechanism exists in the case of the NADH:ubiquinone oxidoreductase with the product of the enzyme catalyzed reaction being ubisemiquinone. Alternatively the enzyme may catalyze a $2e^-$ reduction of ubiquinone to the corresponding quinol. If this were so, then it is interesting that a similarity exists in the amino acid sequence at residues 313 to 320 of the *E. coli* NADH:ubiquinone oxidoreductase and the active-site disulphide region of various soluble disulphide reductases,

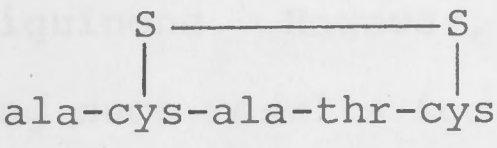
particularly the *E. coli* thioredoxin reductase*.

E. coli NADH:ubiquinone oxidoreductase

ile-gly-asp-cys-ala-ser-cys-pro

(see Chapter 5)

E. coli thioredoxin reductase



(Thelander, 1970)

Thioredoxin reductase, which is a soluble NADPH-specific enzyme consisting of two identical subunits of molecular weight 37,700, accepts four electrons per subunit when fully reduced (two each by the FAD moiety and the disulphide) but alternates between the fully oxidized and $2e^-$-reduced forms during catalysis (Williams, 1976).

The possible involvement of cystine as a second electron-accepting prosthetic group in the *E. coli* NADH:ubiquinone oxidoreductase may be demonstrated by a study of the spectral changes of the pure enzyme following anaerobic titration with NADH. Consistent with the presence of an active site thiol is the finding that the NADH:ubiquinone oxidoreductase activity is reversibly inhibited by Hg^{++} ions (H.D. Campbell, unpublished results).

It is possible to imagine a mechanism involving

---

* This configuration of cysteines is also found in many ferredoxins and rubredoxins (Orme-Johnson, 1973) where, presumably, it fulfils the geometric requirements for co-ordination to Fe/S clusters and Fe atoms respectively.

either a $1e^-$ or a $2e^-$ reduction of ubiquinone to ubisemiquinone or ubiquinol respectively. A study of the mechanism of catalysis of ubiquinone reduction by the purified enzyme will almost certainly provide information as to the mechanism of electron transport in this segment of the respiratory chain, e.g. the possible involvement of ubisemiquinone. However, there is a danger of extrapolating such results too far since electron transport *in vivo* may be different, in detail, to that in the pure enzyme. Thus even though the purified preparation is efficient at catalyzing the reduction of free ubiquinone *in vitro*, the endogenous substrate may be different. King and his co-workers have suggested that ubiquinone participates in mitochondrial electron transport as a protein-bound cofactor, and have isolated a ubiquinone binding protein thought to be involved in the succinate: ubiquinone oxidoreductase reaction (Yu & Yu, 1980, and references therein). They have postulated a similar ubiquinone binding protein for each separate dehydrogenase system including the NADH:ubiquinone oxidoreductase (Yu *et al.*, 1979).

It is evident from the above discussions that the 'plasmid-amplified' strains described in this thesis have proven to be extremely useful in the purification of the *E. coli* NADH:ubiquinone oxidoreductase. The power of such a genetic approach, as used here, is dramatically revealed in Figure 2-6 where identical preparations of NADH: ubiquinone oxidoreductase, from wild-type and genetically-amplified strains, are compared. That such approaches will prove generally applicable, and useful in the

purification of enzymes present in minor amounts in the membrane, is shown by the success in amplifying the levels of the respiratory D-lactate dehydrogenase flavoprotein (see Chapter 3) and, in other work, of phosphatidylserine decarboxylase (Tyhach *et al.*, 1979).

That the defect in the respiratory NADH dehydrogenase in *ndh* mutant strains was corrected by overproduction of the membrane-bound D-lactate dehydrogenase, in strains carrying the hybrid plasmid pIY2, highlights the fact that the considerable elevation of particular enzymes, which can be achieved by gene cloning, can give rise to new metabolic routes which are not significant in wild-type cells. These results suggest that modulation of metabolic pathways using gene cloning provides a new approach to studying such aspects as regulation and rate-limitation in metabolism, as was pointed out elsewhere (Raetz *et al.*, 1977). In this context, since the level of NADH is believed to be important in the regulation of many areas of intermediary metabolism, the plasmids pIY1 and pIY2 may provide interesting insights into the maintenance of the NADH/NAD^+ ratio in the cell. Mention has already been made (see Chapter 3) of the demonstration that the NADH and D-lactate dehydrogenases are rate-limiting for their respective oxidase in wild-type membranes.

The use of strain IY35, in conjunction with the *ndh* mutant strains, has also proven invaluable to the identification of the respiratory NADH dehydrogenase. This was complicated by the fact that there are very high levels of contaminating NADH dehydrogenase activities in

E. coli. From the results presented in Chapter 2, it was found that about 4% of the total NADH-specific ferricyanide reductase and 20% of the NADH-specific ubiquinone-1 reductase activity of crude wild-type extracts is recovered in washed membrane particles: most of the activity appears in the cytoplasmic fraction. As discussed in Chapter 2, there are also multiple NADH dehydrogenase activities in the cell membrane, and this is illustrated by the several NADH:ferricyanide oxidoreductase peaks resolved during hydroxylapatite column chromatography: for apart from the activity associated with the respiratory enzyme, there is a group of NADH-specific ferricyanide reductase peaks eluting in the middle of the gradient and a peak, sometimes resolved into two peaks (*e.g.* Figure 2-5(a)) depending upon whether the column was washed in equilibration buffer prior to commencement of the gradient, at the beginning of the column profile.

As seen from the attempted purification of the mitochondrial NADH:ubiquinone oxidoreductase, gross changes in the catalytic properties of membrane-bound enzymes can accompany their solubilization. Clearly in the case of *E. coli*, where there are multiple NADH dehydrogenase activities in the membrane, a means of assigning the respiratory enzyme in solubilized preparations, which is independent of its catalytic properties, is essential. From a consideration of the column profiles of *ndh* mutant (IY12), wild-type (IY13) and genetically-amplified (IY35 and IY85) strains, it is possible not only to definitively identify the

respiratory enzyme from amongst the activities resolved, but also to state unequivocally that the other activities are not linked to the respiratory chain.

In early attempts to purify the respiratory NADH dehydrogenase of *E. coli* either from crude extracts (Wosilait & Nason, 1954; Bragg, 1965) or small particles (Kashket & Brodie, 1963(b); Bragg & Hou, 1967(a) & (b); Gutman *et al.*, 1968) no indications were given as to the purity of the preparations, nor were there adequate criteria to assign the activities purified to the respiratory NADH dehydrogenase. Recently, Dancey *et al.*, (1976) reported the purification of an NADH dehydrogenase from *E. coli* which is active with a variety of electron acceptors including DCIP and ferricyanide, although no activity towards ubiquinone was reported. The most highly purified preparations consisted of a single polypeptide, molecular weight 38,000. The enzyme was purified 16-fold relative to the activity present after solubilization of spheroplasts with 5% (w/v) Triton X-100. The solubilization procedure, however, resulted in the loss of most of the activity originally present in the spheroplasts. From the data presented (Dancey *et al.*, 1976(a)) it appears that there was only a 5 to 6% recovery of DCIP reductase activity after solubilization. The specific activity of the purified enzyme, 0.32μmol DCIP reduced min^{-1} mg^{-1} at 30°, pH7.5, is only 3-fold higher than that of the spheroplasts. As the authors point out, relating the purified enzyme to the activity present before solubilization, to calculate recovery, is complicated by the presence in the membrane of more than one NADH dehydrogenase. But even so, it is

clear that considerable losses of activity would have to have occurred due to their purification procedure.

Because of the very low specific activity of the preparation of Dancey *et al.*, (1976) it is possible that the enzymic activity resides with a minor component in the preparation rather than with the 38,000 molecular weight polypeptide itself. This may be unlikely, but is supported by two lines of evidence: (1) antibody preparations directed against the purified enzyme react strongly with an antigen shown to be identical with a major structural protein present in outer membranes of *E. coli* (Owen & Kaback, 1979). This protein has been shown to have a subunit molecular weight of 37,000 (Garten *et al.*, 1975) and is a major protein component of vesicles prepared by osmotic shock. It appears therefore to be a contaminant in the preparation of Dancey *et al.* and to constitute at least part of the 38,000 molecular weight polypeptide seen. (2) In a later abstract from the same laboratory, Thomson & Shapiro (1979) have described a preparation of NADH:ubiquinone oxidoreductase which has a specific activity of 75μmol min^{-1} mg^{-1} with 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone as electron acceptor, and consists of two polypeptides of molecular weight 37,000 and 46,000. The authors conclude that the NADH dehydrogenase activity is associated with the 37,000 molecular weight polypeptide (from their earlier studies) and that the 46,000 molecular weight polypeptide confers specificity for ubiquinone reduction. However in view of the finding that the

respiratory enzyme has an apparent molecular weight of ~45,000 (this thesis, Young *et al.*, 1978) the possibility must at least be considered that the catalytic activity lies with the 46,000 molecular weight polypeptide and that either small amounts of this are responsible for the activity observed in the earlier preparation of Dancey *et al.*, (1976) or that the 38,000 species is a separate enzyme or proteolytic degradation product of the respiratory NADH dehydrogenase.

It is not possible to unequivocally define the relationship between the 38,000 molecular weight polypeptide purified by Dancey *et al.*, (1976) and the respiratory NADH dehydrogenase, especially in the absence of amino acid composition or sequence data on the former. The difference in subunit molecular weight estimates by SDS gel electrophoresis, even allowing for the different gel systems used in the two laboratories, makes it unlikely that the two polypeptides are identical. If the 38,000 species is the respiratory NADH dehydrogenase, however, the preparation has grossly altered catalytic properties.

Owen & Kaback (1979) have demonstrated the presence of two, or possibly three, antigenically distinct NADH dehydrogenases in the inner membrane of *E. coli* by crossed immunoelectrophoresis of spheroplasts prepared by osmotic shock (antigens 15 and 19/27 in their nomenclature). Two of these (antigens 19 & 27) show lines of partial identity and it is not clear whether they represent separate enzymes. Owen *et al.*, (1980) have assigned antigen 15 as the respiratory NADH dehydrogenase on the

basis that it is located strictly in the membrane. It possesses NADH dehydrogenase activity (in zymograms, using MTT as electron acceptor) and has also been shown to contain Fe (ibid.); the authors note, however, that the Fe may not be associated with the NADH dehydrogenase subunit itself. The other antigen(s) 19/27 has been identified with the enzyme purified by Dancey *et al.*, (1976) on the basis that it reacts, albeit weakly, with antisera prepared against this enzyme (Dancey *et al.*, 1976; Owen & Kaback, 1979; Owen *et al.*, 1980). Neither antigen 19 or 27 contains Fe, and both partition between the membrane and cytoplasm. It remains to be seen which of these antigens, if any, is the respiratory NADH dehydrogenase. This question should be answered by comparing wild-type and genetically-amplified membrane vesicles by crossed immunoelectrophoresis.

The work presented in this thesis describes the first reported purification of the *E. coli* NADH:ubiquinone oxidoreductase, and is possibly the first preparation described of a highly purified, and intact, respiratory-chain-linked NADH:ubiquinone oxidoreductase from any source. A system has been characterized and developed which may prove a good model for studying the basic properties of membrane-bound enzymes. At present the most advanced and widely-studied model for intrinsic membrane proteins is bacteriorhodopsin, which has recently been sequenced (Ovchinnikov *et al.*, 1979; Khorana *et al.*, 1979). This protein may not be a good model for intrinsic membrane proteins in general, however, since it is found

in highly specialized membrane structures, the purple patch, containing only the one type of polypeptide (Oesterhelt & Stoeckenius, 1971).

With the recent advances in genetic engineering techniques, sequence data is starting to become available for intrinsic membrane proteins from DNA sequencing (Büchel *et al.*, 1980; Hensgens *et al.*, 1979; Barrell *et al.*, 1979). Undoubtedly, structural data for intrinsic membrane proteins will be derived mainly from gene sequencing methods in the future.

Future studies should address themselves to two major questions: (1) whether the assumption, made above, that this enzyme contains all the components of the membrane-bound NADH:ubiquinone oxidoreductase is valid* and (2) what is the biological role of this enzyme. There is at the moment no obvious conclusive experimental test of the first question: however experiments may be designed to show (a) whether the enzyme can reduce ubiquinone-8 in artificial membrane vesicles and (b) by designing suitable photoaffinity anlogues, whether the respiratory NADH dehydrogenase interacts with ubiquinone *in vivo*. With respect to the second question, a direct role for this enzyme in energy conservation has yet to be demonstrated. Preliminary attempts, in collaboration with Dr H.D. Campbell, to demonstrate proton gradient formation

---

* Recently a number of respiratory enzymes have been purified from microorganisms which are simpler in their subunit structure than the corresponding enzyme complexes from mitochondria. These include a single subunit cytochrome oxidase from P53 (Sone *et al.*, 1979) and two-subunit cytochrome oxidases from *P. denitrificans* (Ludwig & Schatz, 1980) and *T. thermophilus* (Fee *et al.*, 1980).

in phospholipid vesicles inlaid with pure enzyme by the cholate dialysis procedure (Ragan & Hinkle, 1975) have so far failed. Unless a positive result is obtained it is not clear whether additional components are required for the associated energy conservation reaction or that no site of energy transduction exists in this segment of the respiratory chain.

REFERENCES

Albracht, S.P.J. (1980) *Biochim. Biophys. Acta 612*, 11-28.

Alexander, K., & Young, I.G. (1978(a)) *Biochemistry 17*, 4745-4750.

Alexander, K., & Young, I.G. (1978(b)) *Biochemistry 17*, 4750-4755.

Ames, G. (1968) *J. Bacteriol. 95*, 833-843.

Ashcroft, J.R., & Haddock, B.A. (1975) *Biochem. J. 148*, 349-352.

Bachman, B.J., & Low, K.B. (1980) *Microbiol. Rev. 44*, 1-56.

Barrell, B.G., Anderson, S., Bankier, A.T., de Bruijn, M.H.L., Chen, E., Coulson, A.R., Drovin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R., & Young, I.G. (1980) *Proc. Natl. Acad. Sci. 77*, 3164-3166.

Baum, R.H., & Dolin, M.I. (1965) *J. Biol. Chem. 240*, 3425-3433.

Beinert, H. (1978) *Methods Enzymol. 54E*, 435-445.

Bessey, O.A., Lowry, O.H., & Love, R.H. (1949) *J. Biol. Chem. 180*, 755-769.

Bigelow, C.C. (1967) *J. Theor. Biol. 16*, 187-211.

Bishop, D.H.L., Pandya, K.P., & King, H.K. (1962) *Biochem. J. 83*, 606-614.

Borck, K., Beggs, J.D., Brammar, W.J., Hopkins, A.S., & Murray, N.E. (1976) *Molec. gen. Genet. 146*, 199-207.

Bragg, P.D. (1965) *Biochim. Biophys. Acta 96*, 263-271.

Bragg, P.D., & Hou, C. (1967(a)) *Arch. Biochem. Biophys. 119*, 194-201.

Bragg, P.D., & Hou, C. (1967(b)) *Arch. Biochem. Biophys. 119*, 202-208.

Büchel, D.E., Gronenborn, B., & Müller-Hill, B. (1980) *Nature 283*, 541-545.

Butlin, J.D., Cox, G.B., & Gibson, F. (1971) *Biochem. J. 124*, 75-81.

Capaldi, R.A. (1974) *Arch. Biochem. Biophys. 163*, 99-105.

Capaldi, R.A., & Vanderkooi, G. (1972) *Proc. Natl. Acad. Sci. 69*, 930-932.

Cardenas, J., & Mortenson, L.E. (1974) *Anal. Biochem. 60*, 372-381.

Castor, L.N., & Chance, B. (1959) *J. Biol. Chem. 234*, 1587-1592.

Chance, B. (1961) in Haematin Enzymes (Falk, J.E., Lemberg, R. and Morton, R.K. Eds.) p433, Pergamon Press, London.

Chance, B., & Redfearn, E.R. (1961) *Biochem. J. 80*, 632-644.

Chappell, J.B. (1964) *Biochem. J. 90*, 225-237.

Clarke, L., & Carbon, J. (1976) *Cell 9*, 91-99.

Clewell, D.B. (1972) *J. Bacteriol. 110*, 667-676.

Cohn, E.J., & Edsall, J.T. (1943) in Proteins, Amino Acids and Peptides (Cohn, E.J., & Edsall, J.T. Eds.) pp370-381, Reinhold Pub. Co., NY.

Coles, C.J., Holm, R.H., Kurtz, D.M., Orme-Johnson, W.H., Rawlings, J., Singer, T.P., & Wong, G.B. (1979) *Proc. Natl. Acad. Sci. 76*, 3805-3808.

Collins, C.J., Jackson, D.A., & de Vries, A.J. (1976) *Proc. Natl. Acad. Sci. 73*, 3838-3842.

Cooper, S., & Helmstetter, C.E. (1968) *J. Mol. Biol. 31*, 519-540.

Cox, G.B., Newton, N.A., Gibson, F., Snoswell, A.M., & Hamilton, J.A. (1970) *Biochem. J. 117*, 551-562.

Crane, F.L., & Barr, R. (1971) *Methods Enzymol. 18C*, 137-165.

Cremona, T., & Kearney, E.B. (1963) *Nature 200*, 542-544.

Cremona, T., Kearney, E.B., Villavicencio, M., & Singer, T.P. (1963) *Biochem. Z. 338*, 407-442.

Crestfield, A.M., Moore, S., & Stein, W.H. (1963) *J. Biol. Chem. 238*, 622-627.

Crowder, S.E., & Ragan, C.I. (1977) *Biochem. J. 165*, 295-301.

Dancey, G.F., Levine, A.E., & Shapiro, B.M. (1976) *J. Biol. Chem. 251*, 5911-5920.

Dancey, G.F., & Shapiro, B.M. (1976) *J. Biol. Chem. 251*, 5921-5928.

Darnall, D.W., & Klotz, I.M. (1975) *Arch. Biochem. Biophys. 166*, 651-682.

Davis, K.A., & Hatefi, Y. (1971) *Biochemistry 10*, 2509-2516.

Davis, K.A., Hatefi, Y., Crawford, I.P., & Baltscheffsky, H. (1977) *Arch. Biochem. Biophys. 180*, 459-464.

Davis, K.A., Hatefi, Y., Poff, K.L., & Butler, W.L. (1973) *Biochim. Biophys. Acta 325*, 341-356.

de Otamendi, M.E.D., & Stoppani, A.O.M. (1974) *Arch. Biochem. Biophys. 165*, 21-33.

Doeg, K.A., & Ziegler, D.M. (1962) *Arch. Biochem. Biophys. 97*, 37-40.

Dooijewaard, G., de Bruin, G.J.M., van Dijk, P.J., & Slater, E.C. (1978) *Biochim. Biophys. Acta 501*, 458-469.

Downie, J.A., & Cox, G.B. (1978) *J. Bacteriol. 133*, 477-484.

Dunphy, P.J., & Brodie, A.F. (1971) *Methods Enzymol. 18C*, 407-461.

Easley, C.W. (1965) *Biochim. Biophys. Acta 107*, 386-388.

Enoch, H.G., & Lester, R.L. (1975) *J. Biol. Chem. 250*, 6693-6705.

Ernster, L., Lee, I.Y., Norling, B., & Persson, B. (1969(a)) *FEBS Lett. 3*, 21-26.

Ernster, L., Lee, I.Y., Norling, B., & Persson, B., (1969(b)) *Eur. J. Biochem. 9*, 299-310.

Fee, J.A., Choc, M.G., Findling, K.L., Lorence, R., & Yoshida, T. (1980) *Proc. Natl. Acad. Sci. 77*, 147-151.

Frank, L., & Rybicki, P. (1961) *Arch. Biochem. Biophys. 95*, 441-449.

Futai, M. (1973) *Biochemistry 12*, 2468-2474.

Futai, M., & Kimura, H. (1977) *J. Biol. Chem. 252*, 5820-5827.

Galante, Y.M., & Hatefi, Y. (1979) *Arch. Biochem. Biophys. 192*, 559-568.

Garten, W., Hindennach, I., & Henning, U. (1975) *Eur. J. Biochem. 60*, 303-307.

Gelfand, D.H., Shepard, H.M., O'Farrell, P.H. & Polisky, B. (1978) *Proc. Natl. Acad. Sci. 75*, 5869-5873.

Gibson, F., & Cox, G.B. (1973) *Essays Biochem. 9*, 1-29.

Gregolin, C., & Singer, T.P. (1963) *Biochim. Biophys. Acta 67*, 201-218.

Gross, E. (1967) *Methods Enzymol. 11*, 238-255.

Gutman, M., Schejter, A., & Avi-dor, Y. (1968) *Biochim. Biophys. Acta 162*, 506-517.

Haddock, B.A., Downie, J.A., & Garland, P.B. (1976) *Biochem. J. 154*, 285-294.

Haddock, B.A., & Jones, C.W. (1977) *Bacteriol. Rev. 41*, 47-99.

Haddock, B.A., & Schairer, H.U. (1973) *Eur. J. Biochem. 35*, 34-45.

Hamer, D.H., & Thomas, C.A. (1976) *Proc. Natl. Acad. Sci. 73*, 1537-1541.

Harrison, D.E.F., & Loveless, J.E. (1971) *J. Gen. Microbiol. 68*, 35-43.

Hatefi, Y. (1978) *Methods Enzymol. 53*, 3-4.

Hatefi, Y., Davis, K.A., Baltscheffsky, H., Baltscheffsky M., & Johansson, B.C. (1972) *Arch. Biochem. Biophys. 152*, 613-618.

Hatefi, Y., Haavik, A.G., & Griffiths, D.E. (1961) *Biochem. Biophys. Res. Comm. 4*, 441-446.

Hatefi, Y., Haavik, A.G., & Griffiths, D.E. (1962) *J. Biol. Chem. 237*, 1676-1680.

Hatefi, Y., & Rieske, J.S. (1967) *Methods Enzymol. 10*, 235-239.

Hatefi, Y., & Stempell, K.E. (1969) *J. Biol. Chem. 244*, 2350-2357.

Hatefi, Y., & Stigall, D.L. (1976) in *The Enzymes* (Boyer, P.D. Ed.) Vol.XIII, pp175-297, Academic Press, N.Y.

Hederstedt, L., Holmgren, E., & Rutberg, L. (1979) *J. Bacteriol. 138*, 370-376.

Hemmerich, P., Ehrenberg, A., Walker, W.H., Eriksson, L.E.G., Salach, J., Bader, P., & Singer, T.P. (1969) *FEBS Lett. 3*, 37-40.

Hempfling, W.P. (1970) *Biochim. Biophys. Acta 205*, 169-182.

Hendler, R.W., & Burgess, A.H. (1974) *Biochim. Biophys. Acta* 357, 215-230.

Hensgens, L.A.M., Grivell, L.A., Borst, P., & Bos, J.L. (1979) *Proc. Natl. Acad. Sci. 76*, 1663-1667.

Heron, C., Corina, D., & Ragan, C.I. (1976) *FEBS Lett. 79*, 399-403.

Heron, C., Smith, S., & Ragan, C.I. (1979) *Biochem. J. 181*, 435-443.

Hershfield, V., Boyer, H.W., Yanofsky, C., Lovett, M.A., & Helinski, D.R. (1974) *Proc. Natl. Acad. Sci. 71*, 3455-3459.

Hirs, C.H.W. (1956) *J. Biol. Chem. 219*, 611-621.

Itagaki, E., & Hager, L.P. (1966) *J. Biol. Chem. 241*, 3687-3695.

Jackson, D.A., Symons, R.H., & Berg, P. (1972) *Proc. Natl. Acad. Sci. 69*, 2904-2909.

Johnsrud, L. (1978) *Proc. Natl. Acad. Sci. 75*, 5314-5318.

Jones, C.W., & Redfearn, E.R. (1966) *Biochim. Biophys. Acta 113*, 467-481.

Kaback, H.R. (1971) *Methods Enzymol. 22*, 99-120.

Kaniuga, Z. (1963) *Biochim. Biophys. Acta 73*, 550-564.

Kashket, E.R., & Brodie, A.F. (1963(a)) *Biochim. Biophys. Acta 78*, 52-65.

Kashket, E.R., & Brodie, A.F. (1963(b)) *J. Biol. Chem. 238*, 2564-2570.

Khorana, H.G., Gerber, G.E., Herlihy, W.C., Gray, C.P., Anderegg, R.J., Nihei, K., & Biemann, K. (1979) *Proc. Natl. Acad. Sci. 76*, 5046-5050.

King, T.E., & Howard, R.L. (1962) *J. Biol. Chem. 237*, 1686-1698.

Kistler, W.S., Hirsch, C.A., Cozarelli, N.R., & Lin, E.C.C. (1969) *J. Bacteriol. 100*, 1133-1135.

Kline, E.S., & Mahler, H.R. (1965) *Ann. N.Y. Acad. Sci. 119*, 905-909.

Kohn, L.D., & Kaback, H.R. (1973) *J. Biol. Chem. 248*, 7012-7017.

Koslov, J.I., Kalinina, N.A., Gening, L.V., Rebentish, B.A., Strongin, A.Y., Bogush, V.G., & Debabov, V.G. (1977) *Molec. gen. Genet. 150*, 211-219.

Kröger, A., & Klingenberg, M. (1966) *Biochem. Z. 344*, 317-336.

Kröger, A., & Klingenberg, M. (1970) *Vitamins & Hormones 28*, 533-574.

Kröger, A., & Klingenberg, M. (1973(a)) *Eur. J. Biochem. 34*, 358-368.

Kröger, A., & Klingenberg, M. (1973(b)) *Eur. J. Biochem. 39*, 313-323.

Kumar, S.A., Rao, N.A., Felton, S.P., & Huennekens, F.M. (1968) *Arch. Biochem. Biophys. 125*, 436-446.

Laemmli, U.K. (1970) *Nature 227*, 680-685.

Lawford, H.G., & Haddock, B.A. (1973) *Biochem. J. 136*, 217-220.

Lehninger, A.L. (1964) in *The Mitochondrion*, Benjamin Inc., N.Y.

Lester, R.L., & Fleischer, S. (1959) *Arch. Biochem. Biophys. 80*, 470-473.

Lin, E.C.C., Koch, J.P., Chused, T.M., & Jorgensen, S.E. (1962) *Proc. Natl. Acad. Sci. 48*, 2145-2156.

Lobban, P.E., & Kaiser, A.D. (1973) *J. Mol. Biol. 78*, 453-471.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951) *J. Biol. Chem. 193*, 265-275.

Ludwig, B., & Schatz, G. (1980) *Proc. Natl. Acad. Sci. 77*, 196-200.

Machinist, J.M., & Singer, T.P. (1965) *Proc. Natl. Acad. Sci. 53*, 467-474.

Mackler, B. (1961) *Biochim. Biophys. Acta 50*, 141-146.

Mahler, H.R., Sarkar, N.K., Vernon, L.P., & Alberty, A.R. (1952) *J. Biol. Chem. 199*, 585-597.

Mendez, E., & Lai, C.Y. (1975) *Anal. Biochem. 68*, 47-53.

Merola, A.J., Coleman, R., & Hansen, R. (1963) *Biochim. Biophys. Acta 73*, 638-640.

Michl, H. (1951) *Monatsch. Chem. 82*, 489-496.

Moore, S. (1963) *J. Biol. Chem. 238*, 235-237.

Oesterhelt, D., & Stoeckenius, W. (1971) *Nature 233*, 149-152.

Ohnishi, T. (1975) *Biochim. Biophys. Acta 387*, 475-490.

Ohnishi, T., Leigh, J.S., Ragan, C.I., & Racker, E. (1974) *Biochem. Biophys. Res. Comm. 56*, 775-781.

Ohnishi, T., Salerno, J.C., Winter, D.B., Lim, J., Yu, C.A., Yu, L., & King, T.E. (1976) *J. Biol. Chem. 251*, 2094-2104.

Olsiewski, P.J., Kaczorowski, G.J., & Walsh, C. (1980) *J. Biol. Chem. 255*, 4487-4494.

Olson, S.T., & Massey, V. (1979) *Biochemistry 18*, 4714-4724.

Orme-Johnson, W.H. (1973) *Ann. Rev. Biochem. 42*, 159-205.

Orme-Johnson, N.R., Hansen, R.E., & Beinert, H. (1974) *J. Biol. Chem. 249*, 1922-1927.

Orme-Johnson, N.R., Orme-Johnson, W.H., Hansen, R.E., Beinert, H., & Hatefi, Y. (1971) *Biochem. Biophys. Res. Comm. 44*, 446-452.

Ovchinnikov, Yu. A., Abdulaev, N.G., Feigina, M.Yu., Kiselev, A.V., & Lobanov, N.A. (1979) *FEBS Lett. 100*, 219-224.

Owen, P., & Kaback, H.R. (1979) *Biochemistry 18*, 1413-1422.

Owen, P., Kaczorowski, G.J., & Kaback, H.R. (1980) *Biochemistry 19*, 596-600.

Pandya, K.P., & King, H.K. (1966) *Arch. Biochem. Biophys. 114*, 154-157.

Penke, B., Ferenczi, R., & Kovács, K. (1974) *Anal. Biochem. 60*, 45-50.

Pharo, R.L., Sordahl, L.A., Vyas, S.R., & Sanadi, D.R. (1966) *J. Biol. Chem. 241*, 4771-4780.

Poole, R.K. & Haddock, B.A. (1974) *Biochem. J. 144*, 77-85.

Poole, R.K., & Haddock, B.A. (1975) *Biochem. J. 152*, 537-546.

Portis, A., Newton, C., Pangborn, W., & Papahadjopoulos, D. (1979) *Biochemistry 18*, 780-790.

Pratt, E.A., Fung, L.W.M., Flowers, J.A., & Ho, C. (1979) *Biochemistry 18*, 312-316.

Pudek, M.R., & Bragg, P.D. (1974) *Arch. Biochem. Biophys. 164*, 682-693.

Pudek, M.R., & Bragg, P.D. (1975) *FEBS Lett. 50*, 111-113.

Rabinowitz, J. (1978) *Methods Enzymol. 53*, 275-277.

Raetz, C.R.H., Larson, T.J., & Dowhan, W. (1977) *Proc. Natl. Acad. Sci. 74*, 1412-1416.

Ragan, C.I. (1976(a)) *Biochim. Biophys. Acta 456*, 249-290.

Ragan, C.I. (1976(b)) *Biochem. J. 154*, 295-305.

Ragan, C.I., & Hinkle, P.C. (1975) *J. Biol. Chem. 250*, 8472-8476.

Ragan, C.I., & Racker, E. (1973) *J. Biol. Chem. 248*, 2563-2569.

Raunio, R.P., Straus, L.D., & Jenkins, W.T. (1973) *J. Bacteriol. 115*, 567-573.

Raw, I., Nogueira, O.C., & Filho, J.B.M. (1961) *Enzymologica 23*, 123-128.

Redfearn, E.R., & Pumphrey, E.R. (1960) *Biochem. J. 76*, 64-71.

Salach, J., Walker, W.H., Singer, T.P., Ehrenberg, A., Hemmerich, P., Ghisla, S., & Hartman, U. (1972) *Eur. J. Biochem. 26*, 267-278.

Sato, M., Ohishi, N., Nishikimi, M., & Yagi, K. (1977) *Biochem. Biophys. Res. Comm. 78*, 868-873.

Scarpulla, R.C., & Soffer, R.L. (1978) *J. Biol. Chem. 253*, 5997-6001.

Schatz, G., & Racker, E. (1966) *J. Biol. Chem. 241*, 1429-1437.

Schieffer, H.G., & Neuhoff, V. (1971) *Z. Physiol. Chem. 352*, 913-926.

Schryvers, A., Lohmeier, E., & Weiner, J.H. (1978) *J. Biol. Chem. 253*, 783-788.

Shipp, W.S. (1972) *Arch. Biochem. Biophys. 150*, 459-472.

Singer, T.P., Kearney, E.B., & Kenney, W.C. (1973) *Advan. Enzymol. 37*, 189-272.

Singer, T.P., & Gutman, M. (1971) *Advan. Enzymol. 34*, 79-153.

So, M., Gill, R., & Falkow, S. (1975) *Molec. gen. Genet. 142*, 239-249.

Sone, N., Ohyama, T., & Kagawa, Y. (1979) *FEBS Lett. 106*, 39-42.

Steffen, D., & Schleif, R. (1977) *Molec. gen Genet. 157*, 341-344.

Storey, B.T., & Chance, B. (1967) *Arch. Biochem. Biophys. 121*, 279-289.

Stouthamer, A.H., & Bettenhaussen, C. (1973) *Biochim. Biophys. Acta 301*, 53-70.

Strittmatter, P. (1965) *J. Biol. Chem. 240*, 4481-4487.

Stroobant, P., Young, I.G., & Gibson, F. (1972) *J. Bacteriol. 109*, 134-139.

Studier, F.W. (1973) *J. Mol. Biol. 79*, 237-248.

Sugino, Y., Tsunasawa, S., Yutani, K., Ogasahara, K., & Suzuki, M. (1980) *J. Biochem. 87*, 351-354.

Sweetman, A.J., & Griffiths, D.E. (1971) *Biochem. J. 121*, 117-124.

Szarkowska, L. (1966) *Arch. Biochem. Biophys. 113*, 519-525.

Tanner, M.J.A. (1979) in *Current Topics in Membranes and Transport* (Bronner, F., & Kleinzeller, A., Eds.) Vol.12, ppl-51, Academic Press, N.Y.

Tarmy, E.M., & Kaplan, N.O. (1968) *J. Biol. Chem. 243*, 2587-2596.

Thelander, L. (1970) *J. Biol. Chem. 245*, 6026-6029.

Thomson, J.W., & Shapiro, B.M. (1979) *Fed. Proc. 38*, 2157 abs.

Tyhach, R.J., Hawrot, E., Satre, M., & Kennedy, E.P. (1979) *J. Biol. Chem. 254*, 627-633.

van der Beek, E.G., & Stouthamer, A.H. (1973) *Arch. Mikrobiol. 89*, 327-339.

Vapnek, D., Alton, N.K., Bassett, C.L., & Kushner, S.R. (1976) *Proc. Natl. Acad. Sci. 73*, 3492-3496.

Waley, S.G., & Watson, J. (1953) *Biochem. J. 55*, 328-337.

Wallace, B.J., & Young, I.G. (1977(a)) *Biochim. Biophys. Acta 461*, 75-83.

Wallace, B.J., & Young, I.G. (1977(b)) *Biochim. Biophys. Acta 461*, 84-100.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406-4412.

Weiss, H., & Kolb, H.J. (1979) *Eur. J. Biochem. 99*, 139-149.

Weiss, H., & Wingfield, P. (1979) *Eur. J. Biochem. 99*, 151-160.

Wickner, S.H., Wickner, R.B., & Raetz, R.H. (1976) *Biochem. Biophys. Res. Comm. 70*, 389-396.

Williams, C.H. (1976) in *The Enzymes* (Boyer, P.D. Ed.) Vol.XIII, pp89-173, Academic Press, N.Y.

Wosilait, W.D., & Nason, A. (1954) *J. Biol. Chem. 208*, 785-798.

Yamada, S., & Itano, H.A. (1966) *Biochim. Biophys, Acta 130*, 538-540.

Young, I.G., Jaworowski, A., & Poulis, M.I. (1978) *Gene 4*, 25-36.

Young, I.G., & Poulis, M.I. (1978) *Gene 4*, 175-179.

Young, I.G., Stroobant, P., Macdonald, C.G., & Gibson, F. (1973) *J. Bacteriol. 114*, 42-52.

Young, I.G., & Wallace, B.J. (1976) *Biochim. Biophys. Acta 449*, 376-385.

Yu, C.A., & Yu, L. (1980) *Biochemistry 19*, 3579-3585.

Yu, C.A., Yu, L., & King, T.E. (1977(a)) *Biochem. Biophys. Res. Comm. 78*, 259-265.

Yu, C.A., Yu, L., & King, T.E. (1977(b)) *Biochem. Biophys. Res. Comm. 79*, 939-946.

Yu, C.A., Yu, L., & King, T.E. (1979) *Fed. Proc. 38*, 2159 abs.

Ziegler, D.M., & Doeg, K.A. (1959) *Biochem. Biophys. Res. Comm. 1*, 344-349.

Ziegler, D.M., & Doeg, K.A. (1962) *Arch. Biochem. Biophys. 97*, 41-50.